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Particulates Initiate Immune Response Via Inducing Oxidative Stress-Mediated NLRP3 Inflammasome Activation

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LOYOLA UNIVERSITY CHICAGO

**PARTICULATES INITIATE IMMUNE RESPONSE VIA INDUCING
OXIDATIVE STRESS-MEDIATED NLRP3 INFLAMMASOME ACTIVATION**

**A DISSERTATION SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY**

PROGRAM IN MICROBIOLOGY AND IMMUNOLOGY

BY

ZHENYU ZHONG

CHICAGO, ILLINOIS

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To my family

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LIST OF ABBREVIATIONS

ADPR	ADP ribose
AIM2	Absent in melanoma 2
ASC	Apoptosis-associated speck-like protein containing a CARD
ATP	Adenosine triphosphate
cADPR	Cyclic ADP-ribose
CARD	Caspase recruitment domain
CaSR	Calcium sensing receptor
CCL20	Chemokine (C-C motif) ligand 20
CD80	Cluster of differentiation 80
CD86	Cluster of differentiation 86
Chol	Cholesterol
CHOP	ER stress-induced transcription factor C/EBP homologous protein
DC	Dendritic cell
DC-Chol	3 β -[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol hydrochloride
DDA	Dimethyldioctadecylammonium (bromide salt)
DMPG	1,2-dimyristoyl-sn-glycero-3-phospho-(1'-rac-glycerol)
DOPC	1,2-dioleoyl-sn-glycero-3-phosphocholine

DOTAP	1,2-Dioleoyl-3-trimethylammonium-propane
DOTAP-OVA	OVA encapsulated DOTAP liposomes
DPPC	1,2-dipalmitoyl-sn-glycero-3-phosphocholine
dsDNA	double-stranded DNA
dsRNA	double-stranded RNA
ER	Endoplasmic reticulum
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HMGB1	High mobility group box 1
hr	Hour
IL-18	Interleukin 18
IL-1R	Interleukin 1 receptor
IL-1 β	Interleukin 1 beta
LPS	Lipopolysaccharide
LRRs	Leucine-rich repeats
M-CSF	Macrophage colony stimulating factor
MAVS	Mitochondrial anti-viral signaling protein
MHR	N-terminus homology domain
min	Minute
ml	Milli-liter
mM	Milli-molar

MSU	Monosodium urate
mtDNA	Mitochondrial DNA
NACHT	Nucleotide-binding and oligomerization domain
NLR	Nod-like receptor
NLRC4	Nod-like receptor family, CARD domain containing 4
NLRP1	Nod-like receptor family, pyrin domain containing 1
NLRP3	Nod-like receptor family, pyrin domain containing 3
nM	Nanomolar
NOD1	Nucleotide-binding oligomerization domain-containing protein 1
NOD2	Nucleotide-binding oligomerization domain-containing protein 2
NOD3	Nucleotide-binding oligomerization domain-containing protein 3
NOD5	Nucleotide-binding oligomerization domain-containing protein 5
NOX	NADPH oxidase
NUD	Nudix-like domain
OVA	Ovalbumin
PAMP	Pathogen-associated molecular pattern
PARG	poly(ADP-ribose) glycohyrolase
PARP	poly(ADP-ribose) polymerase
PC	L- α -phosphatidylcholine (soy extract)
PRRs	Pathogen recognition receptors
PYD	Pyrin domain
ROS	Reactive oxygen species
Th1	T-helper 1

Th2	T-helper 2
TLR	Toll-like receptor
TM	Transmembrane domain
TRPM2	Transient receptor potential melastatin 2
TRX	Thioredoxin
TXNIP	Thioredoxin interacting protein
VDAC	Voltage dependent anion channel
μg	Microgram
μl	Microliter
μM	Micromolar

ABSTRACT

Liposomes are lipid particles formed by self-aggregated phospholipids. They are clinically used both as delivery vectors and immune adjuvants to treat many human infectious and cancerous diseases. However, how liposomes interact with host immune system remains poorly understood. Particularly, little is known about whether an innate immune receptor exists to sense liposomes. I hypothesize that liposomes activate NLRP3 inflammasome in macrophages, which results in the release of interleukin 1 β (IL-1 β), a proinflammatory cytokine that plays a key role in innate and adaptive immune responses.

To test my hypothesis, I will determine the ability of inducing IL-1 β secretion from macrophages by differentially charged liposomes (i.e. cationic, neutral or anionic liposomes). Furthermore, I will also test whether the intact NLRP3 inflammasome machinery is required for liposomes-induced IL-1 β secretion. Notably, similar to liposomes, accumulating evidence has indicated that exposure to other particulates, such as crystals, can also activate the NLRP3 inflammasome in phagocytes. To delineate the molecular mechanism by which particulates (liposomes/crystals) activate the NLRP3 inflammasome, I further hypothesize that particulates induce oxidative stress in macrophages resulting in the production of mitochondrial reactive oxygen species (ROS). Accumulation of ROS can then induce calcium influx via the transient receptor potential melastatin 2 (TRPM2) ion channel, which results in the drastic increase of intracellular calcium concentration. The change in the ionic environment would create a favorable

condition for activation of the NLRP3 inflammasome. To test this, I plan to use combinations of chemical and genetic approaches. First, I will determine whether liposomes can induce mitochondrial ROS production and subsequent ROS-dependent calcium influx via TRPM2 *in vitro*. Next, by taking advantage of the TRPM2 gene knockout mice, I will further determine if genetic ablation of TRPM2 could impair liposomes/crystals-induce NLRP3 inflammasome activation both *in vitro* and *in vivo*. Lastly, because IL-1 β -IL-1 receptor signaling axis is critical for inducing humoral immune responses, I want to test if blocking this signaling pathway would affect liposomal adjuvant effect *in vivo*. Briefly, I will determine if, in comparison with wild-type animals, IL-1RI deficient mice would have any defect in mounting target-antigen specific antibody responses. Taken together, this project will not only identify the previously unknown innate immune sensor for liposomes, but also provide novel insights of the molecular mechanism underlying particle-induced NLRP3 inflammasome activation.

CHAPTER 1

INTRODUCTION

LIPOSOMES

Liposomes are lipid particles that are formed by self-aggregated phospholipids (Allison and Gregoriadis, 1974; Christensen et al., 2007; Slingerland et al., 2012). The lipids contain a nonpolar portion, typically consisting of fatty acid chains, and a polar portion, typically consisting of a phosphate group and/or tertiary ammonium salts (Allison and Gregoriadis, 1974; Miller et al., 1998; Perrie et al., 2003; Slingerland et al., 2012; Vyas and Khatri, 2007). Based on the different charge of the polar region, liposomes can be further subgrouped into positively charged (cationic), negatively charged (anionic) and neutral liposomes (Christensen et al., 2007; Perrie et al., 2003; Slingerland et al., 2012; Vyas and Khatri, 2007). A large panel of phospholipids can be made into liposomes. Liposomes are useful tools that can be clinically used to treat human diseases. They can be used as drug delivery vector/vesicle in anti-cancer therapy, and they are also very important immune adjuvant because they possess strong immunoactivating properties (Allison and Gregoriadis, 1974; Christensen et al., 2007; Slingerland et al., 2012).

Liposomes are considered as one of the next-generation delivery vectors that are superior to many conventional therapeutic vectors for *in vivo* drug-delivery purpose because the composition and structure of liposomes are highly manipulable (Allison and Gregoriadis, 1974; Christensen et al., 2007; Slingerland et al., 2012). For instance, certain

molecules can be directly incorporated into the lipid-bilayer of liposomes or anchored/attached covalently to the lipids(Christensen et al., 2007; Slingerland et al., 2012; Vyas and Khatri, 2007; Walker et al., 1992). This will maximize the amount of drugs carried by delivery vectors and ensure optimal drug efficacy. The ease of attaching molecules onto the lipid shell is also helpful for target-cell delivery purpose, especially in anti-cancer therapy where cancer cell-directed drug delivery is highly desirable(Slingerland et al., 2012). Additionally, in comparison with other next-generation delivery vectors, such as synthetic polymeric nanoparticles, liposomes can be easily made into multilamellar liposome, where multiple layers of lipids within the single liposomes can trap more abundant drugs to ensure gradual and constant release of therapeutic agents entrapped within each layer.(Zucker et al., 2009) It has been shown that utilization of multilamellar liposome can substantially improve the anti-cancer therapeutic efficacy(Pagano, 1978; Pagano and Weinstein, 1978; Satish and Surolia, 2002; Segota and Tezak, 2006).

From an immunologic point of view, liposomes are also ideal immune adjuvant because they are more potent than the conventional adjuvant, such as alum, at amplifying the adaptive immune responses against co-administered antigens(Allison and Gregoriadis, 1974; Christensen et al., 2007). Interestingly, it appears that the chemical properties of lipids largely determine the immunologic use of liposomes. Particularly, the surface charge of liposomes has a major influence on the differential ability to activate the immune system(Allison and Gregoriadis, 1974; Christensen et al., 2007). In contrast to the neutral ones, charged liposomes (either cationic or anionic liposomes) are more

commonly used as immune adjuvants because they seem to possess stronger ability to facilitate the optimal induction of antigen-specific immunity. For instance, the DOTAP liposomes, DC-Chol liposomes, and DDA liposomes are all ideal immune adjuvants and they have been shown to be able to significantly amplify antigen-specific immunity. In contrast to charged liposomes, the neutral ones are weak adjuvants (Allison and Gregoriadis, 1974; Christensen et al., 2007).

DOTAP liposome is one example of cationic liposomes that can be used as immune adjuvants. The co-immunization with target protein/peptide with DOTAP liposomes can elicit CD8⁺ T cell responses with increased cytolytic T cell activity (Cui et al., 2005; Walker et al., 1992). Previous studies have suggested that treatment of DOTAP liposomes can upregulate co-stimulatory molecules (e.g. CD80/86) in antigen presenting cells to facilitate innate immune cell maturation (Satish and Surolia, 2002; Vangasseri et al., 2006). Moreover, DOTAP liposomes are also capable of activating MAPK in dendritic cells (DCs), which might play a role in directing downstream cytokine and chemokine expression (Yan et al., 2007).

DC-Chol liposomes are another type of cationic liposomes that can generate a balanced Th1/Th2 response in several animal models (Brunel et al., 1999; Gao and Huang, 1991; Sanchez et al., 2001). It has been shown that the antibody responses against human inactivated influenza vaccine can be enhanced if DC-Chol liposomes were formulated into the vaccine (Guy et al., 2001). DC-Chol liposomes are also effective mucosal adjuvant and were able to upregulate CCL20 expression in epithelial cells from both vagina and uterus (Hattori et al., 2004). Furthermore, the combination of DC-Chol

liposomes with TLR ligands (such as zymosan, a TLR2 agonist) has been shown to induce maturation of DCs and Langerhans' cells (Cremel et al., 2006).

DDA is another cationic lipid that can form liposomes when heated to above its gel-liquid phase transition temperature (47°C) (Davidsen et al., 2005; Feitosa et al., 2000). It has been shown that immunization of mice with hapten-carrier complexes and DDA liposomes generated hapten-specific delayed-hypersensitivity, suggesting the DDA liposomes are able to induce a cell-mediated immunity (Dailey and Hunter, 1974; Snippe et al., 1977). Furthermore, DDA liposomes have also been used as immune adjuvant in various vaccine formulations against viruses, bacteria and even tumors (Katz et al., 1996; Klinguer-Hamour et al., 2002; Kraaijeveld et al., 1983; Lindblad et al., 1997; Prager and Gordon, 1978). Notably, DDA has been shown to be a safe immune adjuvant that does not appear to have side effect as seen by other immune adjuvants in humans (Hilgers and Snippe, 1992; Stanfield et al., 1973; Veronesi et al., 1970).

Although certain charged liposomes are strong immune adjuvants themselves, they can also be used in combination with other immunomodulators, such as pathogen-associated molecular patterns (PAMPs) molecules (e.g. TLR agonists) or self danger-associated molecular patterns (DAMPs) molecules (e.g. HMGB1, dsRNA, ATP) (Christensen et al., 2007). It has been shown that such combinational approaches can further enhance the adjuvanticity of charged liposomes and be helpful at inducing a desirable Th1/Th2 biased or balanced immune responses, respectively (Christensen et al., 2007; Klinguer-Hamour et al., 2002; Pagano, 1978).

In summary, liposomes are both efficient drug-delivery vectors and powerful immune adjuvants. Their clinical use has been proven to be beneficial for the treatment of many human infectious and cancerous diseases(Allison and Gregoriadis, 1974; Christensen et al., 2007; Pagano, 1978). However, little is known about how liposomes interact with the host immune system. Also, the mechanism underlying the apparently different outcome of such interaction remains elusive. Therefore, it is of utmost importance to study how the host immune system recognizes liposomes and subsequent immune responses after the initial recognition. Furthermore, the better understanding of how different liposomes interact with host immune system will also help to optimize the clinical use of liposomes: in the context of vaccine against infectious diseases, the liposomes that are more potent of activating the immune system may be more preferable, whereas the ones that are weak immune activators may be more suitable for anti-cancer drug delivery purpose.

NOD-LIKE RECEPTORS

Nod like receptors (NLRs) are a group of newly identified innate immune receptors or pathogen recognition receptors (PRRs)(Gross et al., 2011; Ogura et al., 2006; Schroder and Tschopp, 2010). The NLR family members are characterized based upon their signature domains with each member protein, such as the leucine-rich repeats (LRRs), the nucleotide-binding and oligomerization (NACHT) domain, the caspase recruitment domain (CARD) or pyrin (PYD) domain(Gross et al., 2011; Schroder and Tschopp, 2010). The NACHT domain is the only universal domain for all members of

NLRs and it mediates the activation of NLRs in response to their stimuli. Based upon phylogenetic analysis, NLRs can be further divided into 3 subfamilies; NLRPs (NLRP1-14), NODs (NOD1-2, NOD3/NLRC3, NOD4/NLRC5, NOD5/NLRX1, CIITA), and IPAFs (IPAF/NLRC4 and NAIP)(Gross et al., 2011; Ogura et al., 2006; Schroder and Tschopp, 2010).

So far, there are 22 NLRs members identified in human and even more in mouse due to genetic expansion. Like other PRRs, such as Toll-like receptors, the NLRs function to sense the presence of pathogens, foreign intruders, and even “self-danger” signals(Gross et al., 2011; Ogura et al., 2006; Schroder and Tschopp, 2010). For instance, *Nlrp1* recognizes anthrax lethal toxin whereas *Nlrc4* detects bacterial flagellin and components of bacterial type III and IV secretion systems(Gross et al., 2011; Ogura et al., 2006; Schroder and Tschopp, 2010). NOD1 and NOD2 recognize bacterial cell wall breakdowns (mesodiaminopimelic acid and muramyl dipeptide, respectively)(Gross et al., 2011; Ogura et al., 2006; Schroder and Tschopp, 2010). NLRP3 appears to recognize the cytosolic changes within innate immune cells (e.g. macrophages) because it can sense a large diverse pool of activators that are structurally and functionally unrelated to each other(Bulua et al., 2011; Gross et al., 2011; Kepp et al., 2011; Naik and Dixit, 2011; Ogura et al., 2006; Schroder and Tschopp, 2010; Tschopp, 2011; West et al., 2011). Although much progress has been made during the last decade, in contrast to TLRs--the function of which have been extensively studies during the last two decades, little is known regarding the specificity and function of most NLRs.

Upon activation, some of the NLRs forms a large cytosolic protein aggregates, termed “inflammasomes”(Gross et al., 2011; Ogura et al., 2006; Schroder and Tschopp, 2010). It functions to induce caspase-1 autocleavage and activation, which mediates the proteolytic processing of immature IL-1 β into its mature and bioactive form. Additionally, active caspase-1 has also been shown to direct pyroptosis, a specialized cell death that is different from apoptosis or necrosis(Kepp et al., 2010; Miao et al., 2011; Schroder and Tschopp, 2010; Strowig et al., 2012). Although some NLRs can form inflammasomes when they are activated by their cognate ligands/stimuli, the composition of inflammasomes is not identical. For instance, NLRP3 inflammasome formation requires NLRP3, ASC and Caspase-1, whereas some other NLRs, such as NLRP1, do not involve ASC for assembly(Gross et al., 2011; Ogura et al., 2006; Schroder and Tschopp, 2010).

THE NLRP3 INFLAMMASOME

The NLRP3 inflammasome in health and disease

The NLRP3 inflammasome forms by NLRP3, ASC and Caspase-1. Its activation is unique among all PRRs because a large pool of diverse stimuli are known to induce the formation of NLRP3 inflammasome(Gross et al., 2011; Jin and Flavell, 2010; Kepp et al., 2011; Ogura et al., 2006; Schroder and Tschopp, 2010; Tschopp, 2011). Notably, these activators are structurally and functionally unrelated, which makes the activation mechanism of NLRP3 inflammasome more mysterious.

So far, a number of studies have identified more than 30 activators of the NLRP3 inflammasome, and the list is still growing (see **Table 1.1**)(Gross et al., 2011; Jin and Flavell, 2010; Kepp et al., 2011; Tschopp, 2011). The activators of NLRP3 inflammasome range from pathogenic bacteria and viruses (e.g. listeria, adenovirus), to environmental insults (e.g. silica crystals) and self-danger signals (e.g. ATP, uric acid crystals). Although these activators differ significantly in their structure, function, and clinical relevance, it appears that they can transmit common signal(s), via a not yet completely understood mechanism, that ultimately drives the assembly of the NLRP3 inflammasome and leads to the maturation and release of powerful proinflammatory cytokines, such as IL-1 β and IL-18(Kepp et al., 2011; Schroder and Tschopp, 2010; Zhou et al., 2011).

Table 1.1. List of activators of NLRP3 inflammasome

Activator class	Activators
Environmental insults	Ultraviolet light
	Asbestos crystals
	Silica crystals
	Skin irritants
Endogenous danger signals	ATP
	Uric acid crystals
	Glucose
	Amyloid β
	Hyaluronan
Immune adjuvant	Alum
	Complete Freund's adjuvant
Pathogen and its associated molecules	<i>Listeria monocytogenes</i>
	Influenza virus
	Adenovirus
	Sendai virus
	<i>Candida albicans</i>
	<i>Saccharomyces cerevisiae</i>
	Hemozoin
	Bacterial pore-forming toxin

Synthetic materials	Titanium particles
	Polymeric nanoparticles
	PLGA particles
	Gold nanoshell particles

Mutations in the NLRP3 inflammasome machinery, namely the *Nlrp3* gene, are frequently associated with many human diseases (Kingsbury et al., 2011; Strowig et al., 2012; Vandanmagsar et al., 2011; Zaki et al., 2011). This is due to the fact that the dysregulated activation of the NLRP3 inflammasome triggers the release of proinflammatory cytokine, and the elevation of systemic and local concentrations of these cytokines are known to mediate undesirable inflammation associated with many devastating autoinflammatory diseases (see **Table 1.2**).

Besides genetic variance, self metabolic products or environmental insults are also capable of activating the NLRP3 inflammasome (Strowig et al., 2012). For instance, the accumulation of uric acid—a metabolic product—at joints can induce chronic local inflammation and pain, the symptom of which is usually referred as “gouty arthritis” or “gout” (Kingsbury et al., 2011; Martinon et al., 2006; Punzi et al., 2012; Rock et al., 2012; Smith et al., 2011). Similar to uric acid, silica crystal is one of the major causal agents for occupational health problems, such as silicosis. The inhalation of silica particles activates the NLRP3 inflammasome in the airway and lung, sensitizes the epithelial cells and induces chronic inflammation in the upper respiratory tract. If left untreated, many

workers exposed to silica particles may eventually develop pulmonary fibrosis(Cassel et al., 2008; Dostert et al., 2008; Hornung et al., 2008).

Accumulating evidence has indicated that many other metabolic products, which are of non-crystalline nature, also activate the NLRP3 inflammasome to initiate inflammatory responses that significantly contribute to the manifest of clinical problems(Strowig et al., 2012). For instance, the activation of NLRP3 inflammasome by human islet polypeptide has been shown to play a major role in facilitating the development of type II diabetes(Masters et al., 2010). Moreover, the NLRP3 inflammasome has been identified as the missing link that connects high fat containing diet with obesity and insulin resistance. Saturated fatty acid, which is highly enriched in high-fat diet, is the trigger for NLRP3 inflammasome activation. Consequently, released IL-1 β will bind to IL-1R that can interfere with insulin signaling(Wen et al., 2011).

In conclusion, solid evidence has suggested a critical role of NLRP3 inflammasome in a number of autoinflammatory or metabolic diseases. Thus, it is of utmost importance to further investigate the activation mechanism of NLRP3 in the context of various human diseases.

Table 1.2. List of human diseases associated with dysregulated NLRP3 inflammasome activation

Diseases associated with dysregulated NLRP3 inflammasome activation	Activators
Gout	Uric acid crystals
Asbestosis	Asbestos crystals
Silicosis	Silica crystals
Atherosclerosis	Cholesterol crystals
Alzheimer's disease	Amyloid β
Parkinson's disease	α -synuclein
Type II diabetes	Fatty acid and/or self-danger signals
Cancer	Self-danger signals (e.g. ATP, uric acid)
Obesity	Fatty acid and/or self-danger signals
Pathogen-infection	Viruses, bacteria, and/or pathogen associated molecules
Geographic atrophy	Alu RNA
Allergy	Environmental insults (e.g. allergic substances)

Activation mechanisms of the NLRP3 inflammasome

Although many activators of NLRP3 have been identified in the last decades, the precise molecular mechanism that leads to the activation of the NLRP3 inflammasome

remains elusive. This is at least in part due to the lack of a universal pathway that is responsible for NLRP3 inflammasome activation by all its stimuli. However, at least three models have been proposed for activating the NLRP3 inflammasome (Kepp et al., 2011; Schroder and Tschopp, 2010; Tschopp and Schroder, 2010) (see **Figure 1.1.**).

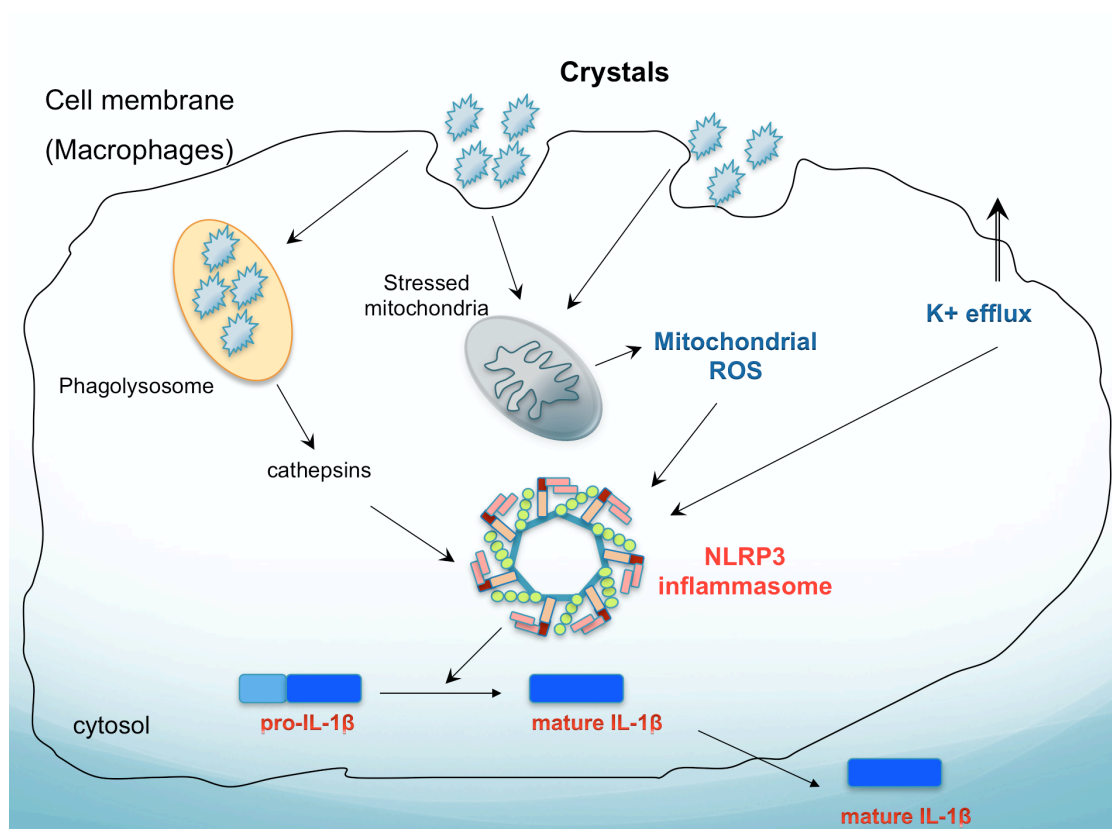


Figure 1.1: Mechanisms of NLRP3 inflammasome activation. Three models have been proposed for NLRP3 inflammasome activation: the first model involves potassium efflux. Activators of NLRP3 inflammasome, such as ATP and crystals, can induce potassium depletion in the cytoplasm, a required step for NLRP3 inflammasome activation; the second model involves rupture of lysosome and subsequent release of lysosomal contents, such as cathepsins. This will somehow lead to the assembly of NLRP3 inflammasome via an unidentified mechanism; the last model involves oxidative stress, in which the mitochondria is damaged upon stimulation of NLRP3 inflammasome activators. This will lead to the accumulation of mitochondrial reactive oxygen species

(ROS), that can somehow transmit signals that directs the assembly of NLRP3 inflammasome. Although a large body of evidence has supported these models respectively, it appears that a unifying model that can incorporate all three mechanisms has yet to be further identified and explored.

The first model involves potassium. In 2007, Tschopp and colleagues first demonstrated that the blockade of potassium efflux could inhibit NLRP3 inflammasome activation (Petrilli et al., 2007). Later studies have also confirmed that potassium efflux is required for NLRP3 inflammasome activation by most, if not all activators (Cassel et al., 2008; Eisenbarth et al., 2008; Gross et al., 2011). Although these data unify a critical role of reducing cytosolic potassium level in driving the assembly of the NLRP3 inflammasome, the mechanism involved remains largely unknown.

The second model for NLRP3 inflammasome activation involves lysosome rupture and subsequent release of lysosomal contents, which somehow induce the activation of the NLRP3 inflammasome. This model seems to apply to most particulate activators because it is conceivable that these large particles may induce phagocytic stress in macrophages, leading to the breakage of lysosome membrane and passive release of lysosomal contents to the cytosol. Latz and colleagues are the first group to identify the lysosomal cathepsin B, a cysteine protease, as a critical player for crystals-induced inflammasome activation (Duewell et al., 2010; Halle et al., 2008; Hornung et al., 2008). They proposed a “lysosome-cathepsin B” model in which the phagocytosed crystals are believed to trigger cathepsin B release from phagolysosome and induce NLRP3 inflammasome assembly and activation. However, their results were solely based on a

cathepsin B inhibitor, CA-074-Me. Consequently, this model was recently challenged based upon the fact the genetic ablation of cathepsin B had little effect on NLRP3 inflammasome activation(Dostert et al., 2009). It appears that the off-target side effect of CA-074-Me is likely to interfere with the data interpretation. In addition to cathepsin B, Latz and colleagues have recently proposed a role for other lysosomal cathepsins, such as cathepsin L, in driving the NLRP3 inflammasome activation(Duewell et al., 2010). However, it remains to be further investigated whether multiple cathepsins play a redundant role in directing NLRP3 inflammasome activation.

The third model involves reactive oxygen species (ROS), which was first brought up by Tschopp and his colleagues in 2008(Dostert et al., 2008). They initially proposed that ROS generated by NADPH oxidase (NOX) during phagocytosis of particles. As ROS are important signaling molecules that can directly or indirectly modify other cellular components or molecules, Tschopp reasoned that ROS might also play a role in NLRP3 inflammasome activation. In fact, the blockade of ROS by anti-oxidant prevented NLRP3 inflammasome activation. However, the “ROS” model was also challenged by others because knocking down of each individual NOXs or their common subunit did not significantly affect NLRP3 inflammasome activation(Bauernfeind et al., 2011; Hornung et al., 2008). In 2011, Tschopp’s group further investigated the source of ROS during NLRP3 inflammasome activation, and they found that ROS are mainly from mitochondria, but not NOXs(Zhou et al., 2011). They elegantly showed solid evidence suggesting that mitochondrial ROS plays a key role in directing the NLRP3 inflammasome activation. Subsequent studies have also found that other contents

released from mitochondria also play a role in NLRP3 inflammasome activation(Misawa et al., 2013; Nakahira et al., 2011). For instance, unoxidized mitochondrial DNA (mtDNA) are shown to activate AIM2 inflammasome which further enhanced the generation of more mature IL-1 β (Nakahira et al., 2011). Additionally, the oxidized mtDNA is able to bind to NLRP3 to directly induce the assembly of NLRP3 inflammasome(Shimada et al., 2012). These findings clearly suggest that mitochondria play a critical role in directing the NLRP3 inflammasome activation. Although mitochondrial ROS is known to signal the assembly of NLRP3 inflammasome, the mechanism involved remains largely unknown.

OXIDATIVE STRESS AND NLRP3 INFLAMMASOME

Mitochondria regulate NLRP3 inflammasome activation

Mitochondrion is an ancient cellular organelle that provides energy for essentially all biological processes within the cell. Notably, studies during the last several decades have clearly suggested additional roles of mitochondria in regulating other pathophysiological events(Tschopp, 2011). For example, a number of neurodegenerative diseases, namely Alzheimer's diseases and Parkinson's diseases, have been closely linked to malfunctioned mitochondria. Additionally, mitochondrial contents and related signaling pathways are key players in shaping the proper immune responses to bacterial and virus infections. MAVS (mitochondrial anti-viral signaling protein) has been one of the best examples for this. Solid evidence has indicated that MAVS plays a key role in

RIG-I like receptor signaling pathway and it regulates antiviral innate immune responses via directing type I interferon production(Hou et al., 2011; Seth et al., 2005).

Recently, Tschopp and colleagues have revealed a novel role of mitochondria in regulating the NLRP3 inflammasome activation(Zhou et al., 2011). They found that blocking of mitochondrial respiratory chain, which results in ROS elevation, could induce spontaneous NLRP3 inflammasome activation in the complete absence of NLRP3 exogenous agonists. Moreover, the mtDNA released from mitochondria upon NLRP3 agonists stimulation also contributes to the proinflammatory responses(Nakahira et al., 2011). Similarly, it has been shown that genetic knocking-down of a mitochondrial inner membrane channel, VDAC (voltage dependent anion channel), attenuates NLRP3 inflammasome activation(Zhou et al., 2011). Later studies have also indicated that the mitochondrial membrane potential is significantly altered upon stimulation with NLRP3 agonists(Barlan et al., 2011; Zhong et al., 2013). This alternation in membrane potential seems to perfectly correlate with the dynamics of NLRP3 inflammasome activation. Nonetheless, it appears that mitochondria can regulate the activation of NLRP3 inflammasome at multiple levels, and the combination of such events ensures the complete activation of the NLRP3 inflammasome. In summary, mitochondria sense the cellular abnormality and transmit signals to induce downstream assembly of the NLRP3 inflammasome and proinflammatory cytokine processing and secretion.

Calcium and NLRP3 inflammasome

Although potassium has been widely accepted as the key player during NLRP3 inflammasome activation (Gross et al., 2011; Schroder and Tschopp, 2010), how cytosolic potassium depletion facilitates the assembly of NLRP3 inflammasome remains largely unknown. In addition to potassium, a role of calcium mobilization has been proposed in NLRP3 inflammasome activation. Horng and her colleagues have shown that blocking calcium flux was able to inhibit NLRP3 inflammasome activation by many NLRP3 agonists, and they further revealed a role of CHOP transcription factor in directing ATP-induced NLRP3 inflammasome activation (Murakami et al., 2012).

Two recent studies have also indicated that extracellular calcium elevation can be recognized by the macrophages as a “self-danger” signal that activates the calcium sensing receptors (CaSR) that can induce an IP₃R-mediated calcium mobilization from ER (Lee et al., 2012; Rossol et al., 2012). This event can, via unknown mechanism, regulate NLRP3 inflammasome activation by many activators.

Despite the evidence indicating a role of calcium in regulating NLRP3 inflammasome activation, little is known regarding how the calcium mobilization is triggered and how the calcium would fit into other known models for NLRP3 inflammasome activation. Particularly, because both mitochondrial ROS and calcium are important for NLRP3 inflammasome activation, how these key players are connected is unclear. Moreover, the identity of potential calcium channel and how elevated calcium induces NLRP3 inflammasome assembly remain to be further investigated.

TRPM2 (TRANSIENT RECEPTOR POTENTIAL MELASTATIN 2) CHANNEL

An overview of the TRPM2 channel

Transient receptor potential (TRP) proteins are comprised of a large family of cation-permeable ion channels that are homologues of the *Drosophila* TRP protein (Link et al., 2010; Sumoza-Toledo and Penner, 2011; Yamamoto et al., 2008). The mammalian TRP superfamily has 28 members, and can be grouped into six subfamilies: canonical or classic (C), polycystin (P), vanilloid (V), melastatin (M), mucolipin (ML) and ankyrin (A). The TRP protein is typically characterized by six-transmembrane domains, with both N- and C-termini oriented toward the cytosolic space. The cation fluxes occur between the fifth and sixth transmembrane domains. The TRP-melastatin subfamily (TRPM), named for melastatin (a tumor suppressor), contains 8 family members: TRPM1–TRPM8. Most TRPM channels (TRPM1–6, and TRPM8) have multiple variants, either as a full-length protein or a short splicing variant, which might explain the complex biological roles of TRPMs, such as modulating the function, ion selectivity and cellular localization of these cation channels. Nonetheless, further study is still needed to delineate the precise function of each channel variants in different cells.

TRPM2 is non-selective multifunctional cation channel, which is Ca^{2+} permeable (Takahashi et al., 2011; Yamamoto et al., 2010). It contains the N-terminus homology domain (MHR), the transmembrane domain (TM) coil-coil domain and C-terminus activation domain (Nudix-like domain, NUDT9-H). The human TRPM2 gene is located in chromosome 21q22.3, and consists of 32 exons. The human TRPM2 gene encodes a protein of 1503 amino acids. Slightly different from its human homologue, the

mouse TRPM2 gene contains 34 exons. The TRPM2 protein comprises six transmembrane segments, flanked by the intracellular N- and C-termini, with the pore-forming channel domain located between the fifth and sixth transmembrane domains.

Although several studies have revealed multiple activators for TRPM2 channel (Takahashi et al., 2011; Yamamoto et al., 2010), such as ADP-ribose (ADPR) (Sumoza-Toledo and Penner, 2011), cyclic ADP-ribose (cADPR), calcium, and H_2O_2 , ADPR has been generally accepted as the primary activating/gating agent of TRPM2 channel. ADPR binds to the NUDT9-H domain on the C-terminus of TRPM2 and is subsequently hydrolyzed to ribose 5-phosphate and AMP, which is most likely due to the enzymatic activity of the C-terminus of TRPM2. Notably, this enzymatic feature of TRPM2 has been proposed to provide a negative regulation mechanism for controlling the opening of TRPM2 because AMP is known to antagonize ADPR-mediated gating of TRPM2 (Sumoza-Toledo and Penner, 2011). Upon binding of TRPM2 gating agents (e.g. ADPR), TRPM2 channels will transiently open and allow the pass through of a number of cations, such as Na^+ , K^+ and Ca^{2+} , into the cell. A linear current–voltage relationship, characterizes TRPM2 currents and TRPM2 has a remarkably long open-time (several seconds) in comparison with other cationic channels (Rossol et al., 2012; Sumoza-Toledo and Penner, 2011; Takahashi et al., 2011).

TRPM2 is expressed in many tissues and organs with its highest level seen in brains (Sumoza-Toledo and Penner, 2011). Due to the unique expression pattern, the role of TRPM2 has been extensively studied in neurons and more importantly, in the context of a number of neurodegenerative diseases, such as Alzheimer's diseases, multiple

sclerosis, and Parkinson's disease. Mice deficient in TRPM2 showed significant protection in animal models of several neurological diseases, suggesting that TRPM2-mediated calcium mobilization positively regulates the onset/progression of such diseases (Naziroglu, 2011).

Besides its expression in brains, TRPM2 has also been detected in other tissues such as spleen, bone marrow, heart, liver and lung, and in a number of different cell types like pancreatic β -cells, endothelial cells, epithelial cells, cardiomyocytes and a panel of immune cells (e.g. neutrophils, monocytes/macrophages and T cells) (Sumoza-Toledo and Penner, 2011; Takahashi et al., 2011). However, in contrast to its role in brain neurons and microglia, the role of TRPM2 in other cells has not yet been largely explored. It is conceivable that the TRPM2-mediated cation fluxes may presumably play regulatory roles in shaping the function of many different cells in which TRPM2 is expressed.

TRPM2 channel and oxidative stress

Due to the fact that TRPM2 channel can open under oxidative stress conditions, much attention has been paid to the role of TRPM2 in sensing the redox change within the cell (Sumoza-Toledo and Penner, 2011; Takahashi et al., 2011). The generation of reactive oxygen species (ROS) is a signature event when cells are under oxidative stress triggered by foreign insults or self-genetic instability. ROS are reactive molecules that are able to modulate almost all other molecules within the cell, such as lipid, protein, DNA, and RNA (Bulua et al., 2011; Kepp et al., 2011; Naik and Dixit, 2011; Tschopp, 2011). Therefore, it has been widely accepted that ROS function as a signaling messenger or

intermediate to direct downstream events which ultimately regulate/alter the activity of certain molecule or the function of a cellular organelle.

Recent studies have linked TRPM2 activity with oxidative stress by showing that gating of TRPM2 channel is frequently associated with oxidative damage in the cell(Takahashi et al., 2011). This notion is supported by the fact that oxidative stress-induced DNA damage in the nucleus is known to induce the accumulation and release of abundant free ADPR, which can bind to TRPM2 to induce cationic fluxes(Naziroglu, 2007; Perraud et al., 2003). Additionally, it has also been proposed that the ADPR released from mitochondria, another source of cellular ADPR, might also contribute to the TRPM2 opening under oxidative stress, because mitochondria are highly sensitive to environmental stress which likely to converts such stress signals to oxidative damage in the cell(Perraud et al., 2005).

From an immunological prospect, TRPM2 is also a critical player in the immune system. For instance, seminal studies by Mori group have clearly demonstrated the key role of TRPM2 in mediating oxidative stress (e.g. ROS) induced upregulation of chemokine expression, which contributes to the aggregation of local proinflammatory responses(Yamamoto et al., 2008). This is of particular interest in innate immunity against microbes because several studies have revealed that mice deficient in TRPM2 are defective in controlling the pathogenesis of bacterial infection. For instance, TRPM2 deficiency has been shown to drastically affect the ability of mouse immune system to restrict *Listeria monocytogenes* infection(Knowles et al., 2011). Interestingly enough, as *Listeria monocytogenes* are known activator of NLRP3 and AIM2 inflammasomes(Kim

et al., 2010), we reasoned it would be interesting to investigate the role of TRPM2-mediated calcium mobilization in inflammasome activation, especially considering the fact that calcium is a critical player for regulating inflammasome activity.

CHAPTER 2

RATIONALE AND DISSERTATION OUTLINE

Liposomes are particles consisting of self-aggregated lipids and are commonly used as effective immune adjuvants and efficient drug delivery vehicles to treat various infectious and cancerous diseases(Al-Jamal and Kostarelos, 2011; Allison and Gregoriadis, 1974; Christensen et al., 2007; Slingerland et al., 2012). Although previous studies have indicated that liposomes can enhance the expression of chemokines, such as CCL2 and co-stimulatory molecules such as CD80/86 in antigen-presenting cells(Cremel et al., 2006; Cui et al., 2005; Vangasseri et al., 2006; Yan et al., 2007), little is known about whether an innate immune receptor exists to sense the presence of liposomes. During the past decade, a number of studies have demonstrated that particulate substances such as alum, silica, monosodium urate, and calcium pyrophosphate dihydrate crystals can induce NLRP3 (Nod-like receptor family, pyrin domain containing 3) inflammasome activation which mediates the secretion of biologically active IL-1 β from macrophages(Dostert et al., 2008; Eisenbarth et al., 2008; Hornung et al., 2008; Li et al., 2008; Martinon et al., 2006). Because liposomes are also of particulate in nature, I hypothesized that the NLRP3 inflammasome may serve as an innate immune sensor for liposomes.

Although the precise mechanism by which the NLRP3 inflammasome is activated remains unknown, several stress-related cellular processes, including cytosolic depletion

of potassium, lysosome disruption, mitochondrial damage and intracellular calcium elevation have been proposed to be involved in activation of this inflammasome (Jin and Flavell, 2010; Murakami et al., 2012). Mitochondria are an ancient and evolutionarily conserved cellular organelle that play a key role in regulating the signaling pathways of pattern recognition receptors (PRRs) (West et al., 2011), including the NLRP3 inflammasome. Upon stimulation with particulate stimuli, mitochondria can transmit stress signals to alarm the immune system via secondary signaling messengers, such as mitochondrial reactive oxygen species (ROS) (Kepp et al., 2011; Tschopp, 2011; West et al., 2011). Blocking ROS generation by mitochondria has been shown to abolish NLRP3 inflammasome activation whereas artificial induction of mitochondrial ROS can spontaneously induce NLRP3-mediated IL-1 β secretion (Nakahira et al., 2011; Zhou et al., 2011). Although it is clear that mitochondrial ROS play a pivotal role, the precise mechanism underlying ROS-induced NLRP3 inflammasome activation remains poorly understood.

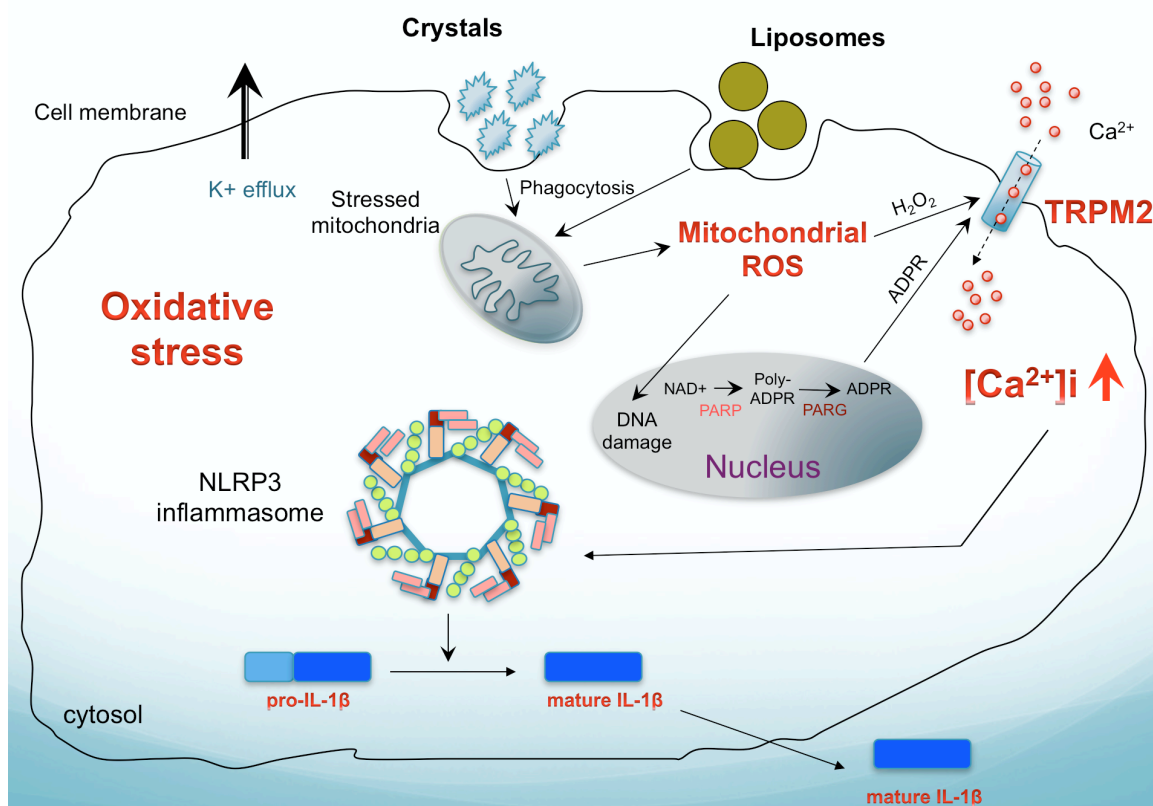


Figure 2.1: Dissertation outline/working model. I hypothesize the charged liposomes, but not neutral ones, activate the NLRP3 inflammsome to induce bioactive IL-1 β secretion from macrophages. Briefly, upon macrophage uptake of charged liposomes, it can induce oxidative stress which makes mitochondria under stressed conditions. This will further lead to the accumulation of mitochondrial reactive oxygen species and subsequently induce a calcium influx via the plasma membrane TRPM2 channel. The gating of TRPM2 channel can be achieved by at least two ways, which depends on free ADP-ribose release from nucleus or direct H₂O₂ opening of the TRPM2 channel, respectively. The elevation in intracellular calcium concentration will then create a preferable ionic environment which favors the assembly of the NLRP3 inflammasome and subsequent caspase-1 activation, proteolytic processing of pro-IL-1 β , and release of bioactive mature IL-1 β . This process will also be accompanied by cytosolic depletion of potassium (potassium efflux). However, the precise relationship between potassium and calcium mobilizations in NLRP3 inflammasome activation process needs further investigation.

CHAPTER 3

MATERIALS AND METHODS

Reagents

1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP), 3 β -[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol hydrochloride (DC-Chol), dimethyldioctadecylammonium (bromide salt) (DDA), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dimyristoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DMPG) and L- α -phosphatidylcholine (soy extract) were purchased from Avanti Polar Lipids (Alabaster, AL). Track-etch polycarbonate membranes (2 μ m, 800 nm, 400 nm, 200 nm and 100 nm) were from Millipore, The Lipex liposomes extruder was from Northern Lipids and other common lab chemicals and reagents were from Sigma-Aldrich and Fisher Scientific. Cholesterol, ovalbumin, silica, ATP, phorbol 12-myristate 13-acetate (PMA), cytochalasin D, uricase, bafilomycin A1, Ca-074-Me, H₂O₂, rotenone, antimycin and BAPTA-AM were from Sigma-Aldrich. Ultrapure LPS, zymosan, and poly(dA:dT), and glibenclamide were from Invivogen. zYVAD-fmk, APDC and DPQ were from Alexis Biochemicals. DPI was from Calbiochem. Monosodium urate crystals, VAS2870 and NAC were from Enzo Lifescience. Inject Alum and streptavidin-HRP were from Pierce. Lipofectamine 2000, MitoSOX and fura-2-AM were from Invitrogen. Calcium free and calcium containing DMEM media were from US Biologicals. The lactate dehydrogenase

(LDH) assay kit was from Roche. Antibodies used for immunoblotting were as follows: anti-mouse caspase-1 (sc-514, Santa Cruz Biotechnology), anti-mouse β -actin (sc-1615 HRP, Santa Cruz Biotechnology) and anti-mouse IL-1 β (AF-401-NA, R&D systems). Antibodies used for determining OVA-specific IgG isotypes IgG1 and IgG2b were from Biolegend, and the antibody for mouse IgG2c was from Bethyl Laboratories.

Liposomes formulations

A total of 16 different liposomes formulations (see Table 3.1) were produced by Encapsula NanoSciences. The liposomes were made using the dehydration-rehydration method. The lipids were dissolved in chloroform in a round bottom flask, and the solvent in the flask was then evaporated using a Büchi (RE-121) rotary evaporator (Flawil, Switzerland) under a vacuum for 4 hours, resulting in the formation of a thin lipid film. The thin lipid film was hydrated with deionized water for 2 hours at 37°C and then overnight at 4°C. The milky solution of liposomes was extruded 25 times through an appropriately sized polycarbonate membrane filter using a Lipex extruder connected to a high-pressure nitrogen cylinder to produce liposomes of the desired sizes. For example, liposomes were passed through a 100-nm polycarbonate membrane filter 25 times to produce 100-nm liposomes.

To formulate ovalbumin-encapsulated liposomes, the thin lipid film was hydrated with a solution of ovalbumin in deionized water. The liposomes were sized to 400 nm. The non-encapsulated ovalbumin was separated using 100K dialysis membrane tubing

(Spectrum Laboratories). The amount of the encapsulated ovalbumin was determined by BCA assay using a BCA protein assay kit (ThermoFisher Scientific).

Table 3.1 Liposome formulations

Lipid composition	Lipid Molar percentage	Size (nm)	Concentration (mg/ml)
DOTAP	100	100	10
DOTAP	100	200	10
DOTAP	100	400	10
DOTAP	100	800	10
DOPC	100	100	10
DOPC	100	200	10
DOPC	100	400	10
DOPC	100	800	10
DOTAP:DOPC	50:50	100	20
DOTAP:DOPC	80:20	100	10
DOTAP:DOPC	90:10	100	10
DPPC:DMPG	70:30	100	10
DC-Cholesterol	100	100	10
DDA	100	100	10
L-α-PC: Cholesterol	70:30	2000	23
DOTAP-OVA (OVA encapsulated)	100	400	10 mg/ml of lipid and 2 mg/ml of encapsulated ovalbumin

Cell culture and stimulation

Human THP-1 cells (ATCC) were grown in RPMI-1640 medium supplemented with 10% (vol/vol) FBS. THP-1 cells were primed with PMA (100 nM) for 3h, washed three times and cultured overnight in serum-free DMEM medium. The cells were then stimulated with either liposomes or crystals for 18h, and the supernatants were collected for the detection of human IL-1 β by ELISA. Bone marrow-derived macrophages (BMDMs) were generated by culturing the mouse bone marrow cells in the presence of

20% vol/vol L929 conditional media as previously described(Hornung et al., 2008; Wen et al., 2011). After pretreatment with ultrapure LPS (100 ng/ml for primary BMDMs and 50 ng/ml for immortalized macrophages) for 16-18h, the BMDMs were stimulated with ATP for 30min unless otherwise indicated or liposomes, alum, silica, MSU crystals or H₂O₂ for 6h. Poly(dA:dT) was transfected into the macrophages using Lipofectamine 2000 (4 µg/ml) according to manufacturer's instructions. For the experiments using chemical inhibitors, the inhibitors were added 45 min prior to stimulation with the NLRP3 inflammasome agonists. The blockade of potassium efflux with a high concentration of KCl was performed as previously described(Eisenbarth et al., 2008). For the experiments using calcium-free medium, BMDMs were first primed with LPS for 18h in calcium-containing DMEM, washed four times with PBS and then cultured in calcium-free DMEM in the presence of inflammasome agonists. Supernatants and cell lysates were collected for ELISA and immunoblotting analyses. Immortalized murine macrophages from *Nlrp3*^{-/-}, *Asc*^{-/-}, *Capase 1*^{-/-}, *Nlrc4*^{-/-}, *Cathepsin B*^{-/-}, *Aim2*^{-/-} mice and their corresponding wild-type control cells were generously provided by Dr. Katherine Fitzgerald and as previously described(Hornung et al., 2008).

Enzyme-linked immunosorbent assay

Paired (capture and detection) antibodies and standard recombinant proteins for mouse IL-1β, tumor necrosis factor and IL-6 (from eBioscience) were used to quantify the cytokine levels in cell culture supernatants according to the manufacturer's instructions. Briefly, 96-well plates were coated with capture antibodies overnight for

each cytokine tested. On the next day, the plates were washed and blocked with 5% non-fat milk dissolved in PBS-T buffer for 2 hours. After washing the plates, dilutions of cell culture supernatants were added into each well and incubated for 2 hours. Detection antibodies (biotin-labeled) were added after washing out the diluted samples, and the plates were incubated for another 2 hours. Then the plates were washed and streptavidin-labeled HRP was added for incubation of 1 hour. The plates were washed and the HRP substrate (TMB) was added and the reaction can be stopped by 1M H₂SO₄. The values of OD₄₅₀ were subsequently measured.

Mitochondrial ROS detection

Mitochondrial ROS levels were measured using MitoSOX (Invitrogen). Briefly, LPS-primed cells were treated with liposomes for 6 h, loaded with 4 μ M of MitoSOX for 15 min and washed twice with sterile PBS. The mean fluorescence intensity was determined using a FACSCanto flow cytometer (BD Bioscience), and the data were analyzed using FlowJo software (Treestar).

Fluorometric measurement of [Ca²⁺]_i

The [Ca²⁺]_i was measured in single cells as previously described (Han et al., 2006). Briefly, cells were loaded for 30 min at 37°C with 2 μ M fura-2 AM in Tyrode solution containing 138 mM NaCl, 2.7 mM KCl, 1.06 mM MgCl₂, 1.8 mM CaCl₂, 5.6 mM glucose and 12.4 mM HEPES (pH 7.4, adjusted with NaOH). The fluorescence emission at 510 nm of fura-2 excited at 340 nm and 380 nm was acquired and analyzed

offline with the NIS-Elements Advanced Research software package (Nikon) using an inverted Nikon Ti-E microscope equipped with a Xenon lamp (Hamamatsu), a 40x 1.30 NA objective (Nikon) and an Evolve 512 EMCCD camera (Photometrics). The EMCCD camera was cooled to -80°C during imaging.

Quantitative real-time PCR analysis

RNA was isolated from murine bone marrow derived macrophages and was reverse transcribed. Quantitative real-time PCR analysis was performed using the StepOnePlus Real-time PCR system (Applied Biosystems). All gene expression data are presented as the expression relative to HPRT1. The primer sequences are described in Table 3.2.

Table 3.2 Primers sequences for real-time PCR analysis.

Mouse gene	Forward	Reverse
<i>Il1b</i>	CTGCAGCTGGAGAGTGTGG	GGGGAACCTCTGCAGACTCAA
<i>Nlrp3</i>	ATGGCTGTGTGGATCTTTGC	CACGTGTCATTCCACTCTGG
<i>Hprt1</i>	CTGGTGAAAAGGACCTCTCG	TGAAGTACTCATTATAGTCAA GGGCA

***In vivo* mouse peritonitis model**

Peritonitis was induced by the intraperitoneal injection of stimuli. Briefly, 6- to 8-week-old mice were intraperitoneally injected with 1 mg MSU crystals, DOTAP liposomes or 0.2 mg zymosan dissolved in 0.5 ml sterile PBS. Six hours after injection, the mice were sacrificed by exposure to CO₂ and the peritoneal cavities were washed with 8 ml cold PBS. The recruited polymorphonuclear neutrophils present in the peritoneal lavage fluid were quantified by flow cytometry using the neutrophil markers Ly6G (BD Bioscience) and CD11b (eBioscience). The samples were acquired on a FACSCanto flow cytometer (BD Biosciences) and the data were analyzed using FlowJo software (Treestar).

Immunization

6- to 8-week-old mice deficient in TRPM2 or IL-1R and the corresponding wild-type controls were subcutaneously immunized with ovalbumin alone (40µg/mouse), ovalbumin-encapsulated DOTAP liposomes or ovalbumin mixed with LPS, and the animals were boosted once after two weeks. Ten days after the final immunization, the mice were sacrificed by exposure to CO₂ and the immune sera from the periphery blood were isolated.

Statistical analyses

All data are shown as mean \pm s.d. or mean \pm s.e.m.. Statistical analysis was performed using a two-tailed Student's *t-test* for all studies. For all tests, *P*-values less than 0.05 were considered statistically significant.

CHAPTER 4

RESULTS

AIM1: DETERMINE WHETHER LIPOSOMES ACTIVATE NLRP3 INFLAMMASOME TO INDUCE BIOACTIVE IL-1 β FROM MACROPHAGES

Charged liposomes, but not neutral ones, induce mature IL-1 β release from macrophages

It is generally accepted that a “two-step” process is necessary for NLRP3 inflammasome-mediated IL-1 β release. The first step requires NF- κ B activation to induce synthesis of pro-IL-1 β , whereas the second step involves assembly of a large cytosolic protein complex, termed inflammasomes, which lead to caspase-1 activation. Caspase-1 then cleaves pro-IL-1 β into its mature form (Gross et al., 2011). To determine whether liposomes can trigger activation of the NLRP3 inflammasome, we first tested whether several liposomes, consisting of different phospholipids, can induce bioactive IL-1 β release in macrophages. Cationic (DOTAP, DC-Chol, and DDA) and anionic (DPPC-DMPG) liposomes elicited IL-1 β release from lipopolysaccharide (LPS)-primed murine bone marrow-derived macrophages (BMDMs) and PMA-primed human THP-1 cells (**Figure 4.1**), and the levels of secreted IL-1 β were dependent on the dose and size of the liposomes (**Figure 4.2**). Procaspace-1 was auto-cleaved after stimulation with charged liposomes, and active caspase-1 was responsible for the maturation of IL-1 β (**Figure 4.3**).

Unlike IL-1 β release, the secretion of both TNF and IL-6, which relies on NF- κ B activation, was not enhanced in LPS-primed BMDMs after liposomes stimulation (**Figure 4.4a, b**). Consistently, liposomes alone did not induce either TNF or IL-6 secretion in unprimed macrophages (**Figure 4.4c**). In sharp contrast to the charged liposomes, neutral liposomes, such as DOPC and PC-Chol liposomes, failed to trigger IL-1 β secretion (**Figure 4.1** and **Figure 4.5a, b**). However, the incorporation of cationic lipids into neutral liposomes rescued IL-1 β secretion (**Figure 4.5c**), which suggests that the charge of liposomes determines their ability to induce IL-1 β release. Furthermore, although liposomes induced low levels of cytotoxicity in macrophages (**Figure 4.6**), ATP and uric acid, two known NLRP3 inflammasome agonists that are released from dying cells (Mariathasan et al., 2006; Martinon et al., 2006), were not intermediates responsible for liposome-induced IL-1 β secretion (**Figure 4.7**).

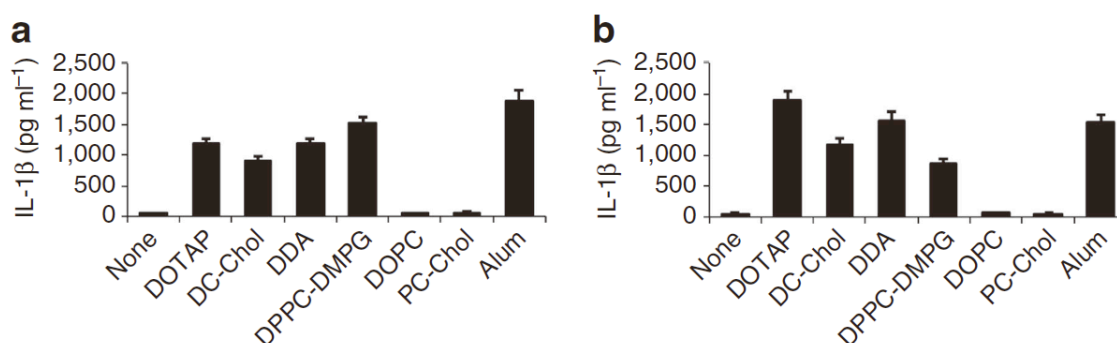


Figure 4.1: Charged liposomes, but not neutral ones, induce IL-1 β secretion from macrophages. ELISA for IL-1 β from the supernatants of **(a)** LPS-primed wild-type BMDMs or **(b)** PMA-primed THP1 cells that were stimulated with either the indicated liposomes (30 μ g/ml for BMDMs and 50 μ g/ml for THP1 cells) or alum (250 μ g/ml for BMDMs and 500 μ g/ml for THP1 cells). Data are shown as mean \pm s.d., and all data are representative of at least three independent experiments.

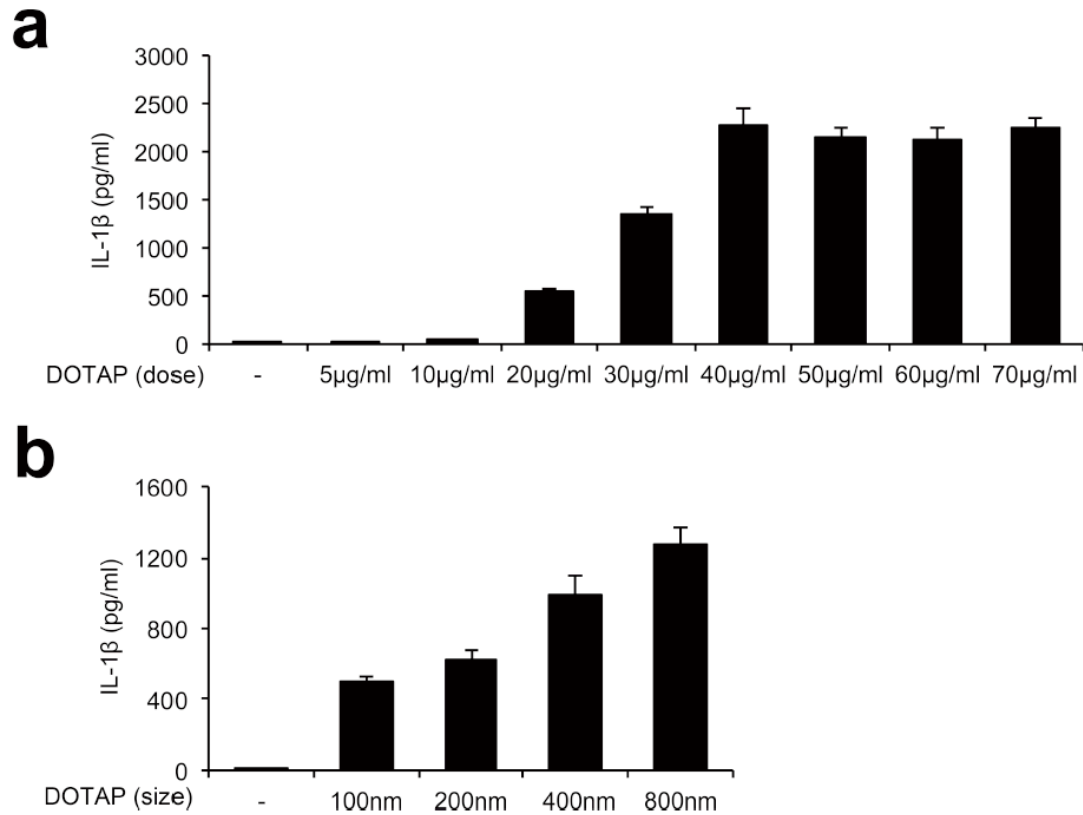


Figure 4.2: Charged liposomes induce IL-1 β secretion in a dose and size dependent manner. ELISA for IL-1 β from supernatants of PMA-primed THP1 cells that were stimulated with DOTAP liposomes of different doses (**a**) or sizes (**b**, 100 nm, 200 nm, 400 nm or 800 nm in diameter, respectively) for 16 h. Data are shown as mean \pm s.d., and are representative of three independent experiments.

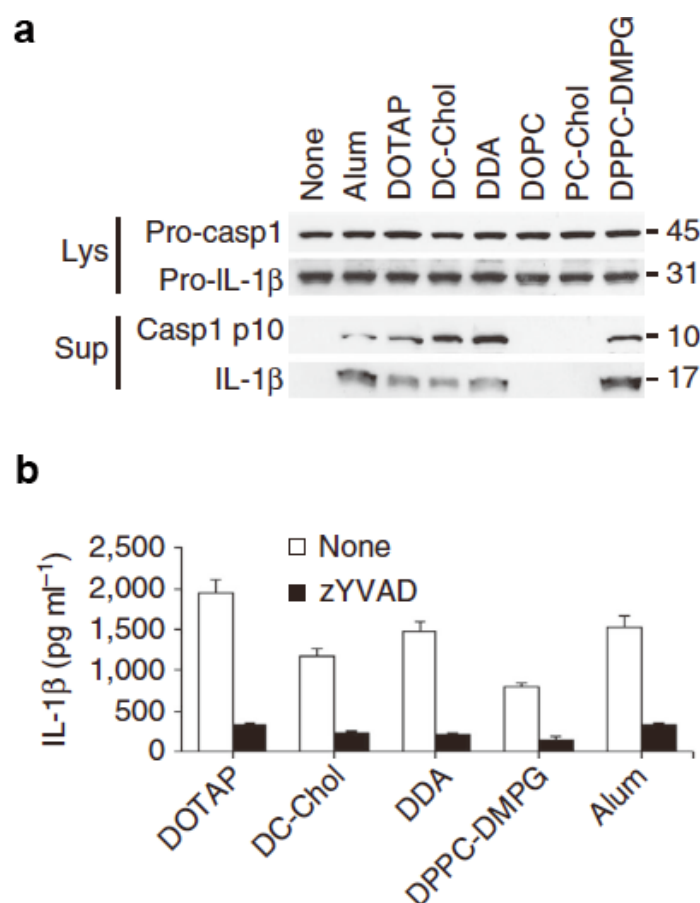


Figure 4.3: Charged liposomes activate caspase-1 to induce bioactive IL-1β release. (a) Immunoblots of procaspase-1, activated caspase-1 (p10), pro-IL-1β and cleaved IL-1β (p17) in the culture supernatants (Sup) and cell lysates (Lys) from LPS-primed BMDMs after stimulation with indicated liposomes or alum. (b) IL-1β from supernatants of LPS-primed BMDMs that were pretreated with caspase-1 inhibitor (z-YVAD-fmk, 10 μM) for 45 min followed by stimulations with indicated liposomes or alum. Data are shown as mean ± s.d. in b, and all data are representative of three independent experiments.

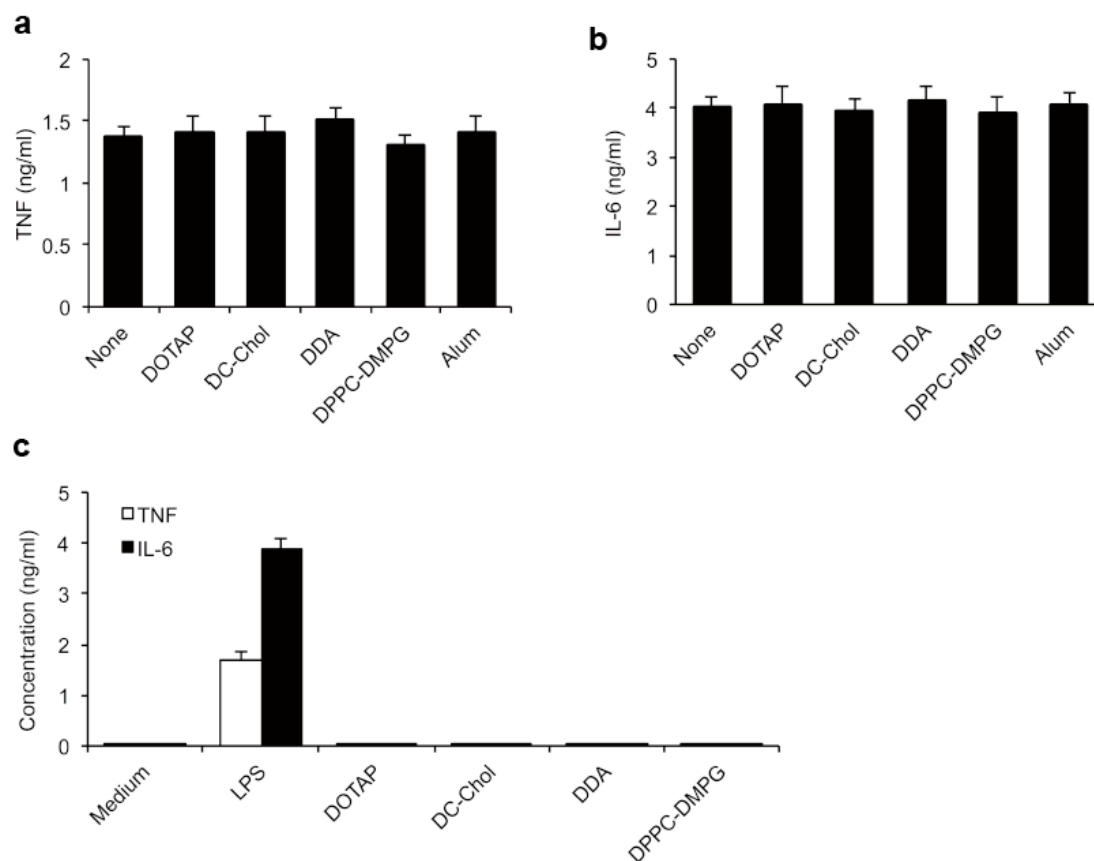


Figure 4.4: Charged liposomes alone do not induce secretions of TNF and IL-6. ELISA for TNF (a) or IL-6 (b) from supernatants of LPS-primed wild-type mouse BMDMs that were stimulated with indicated liposomes. (c) TNF and IL-6 from supernatants of wild-type unprimed BMDMs treated with LPS (100 ng/ml) or indicated liposomes alone (30 μ g/ml). Data are shown as mean \pm s.d., and are representative of three independent experiments.

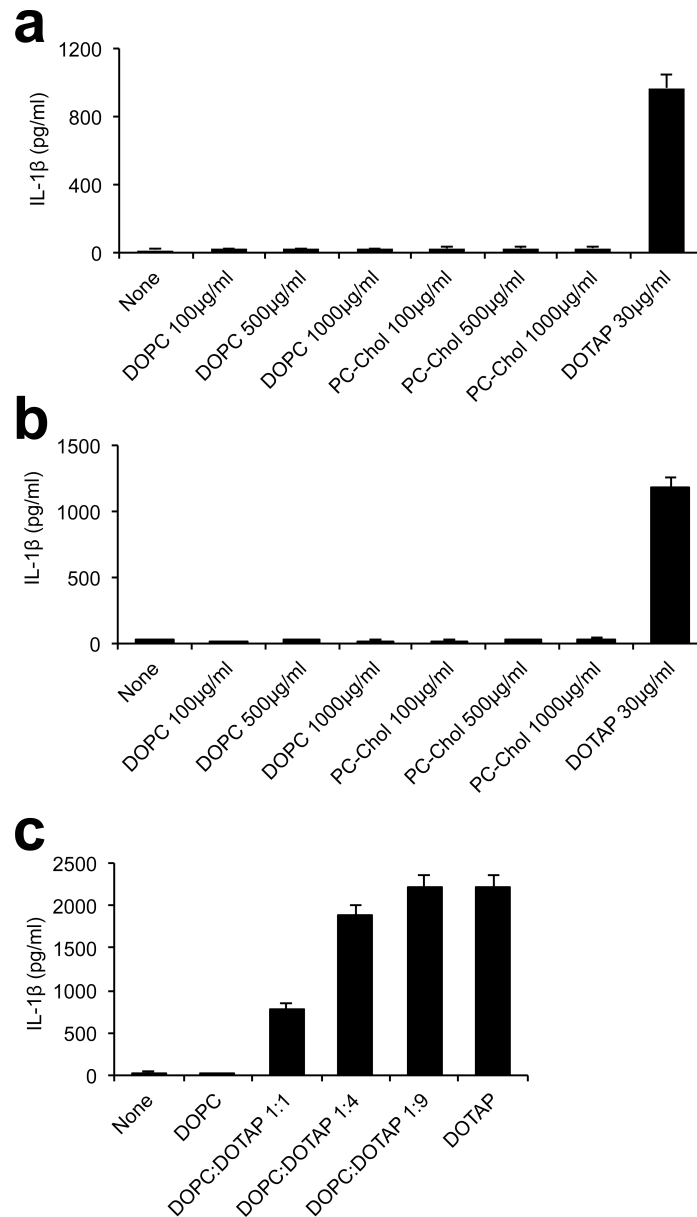


Figure 4.5: Neutral liposomes fail to induce IL-1 β secretion in macrophages. ELISA for IL-1 β from supernatants of LPS-primed wild-type BMDMs (**a**) or PMA-primed THP1 cells (**b**) that were stimulated with indicated neutral liposomes for 24 h. (**c**) ELISA for IL-1 β from supernatants of PMA-primed THP1 cells that were stimulated with pure DOPC or DOTAP liposomes, or chimeric liposomes (50 μ g/ml) made with various molar ratio of DOPC versus DOTAP. Data are shown as mean \pm s.d., and are representative of at least three independent experiments.

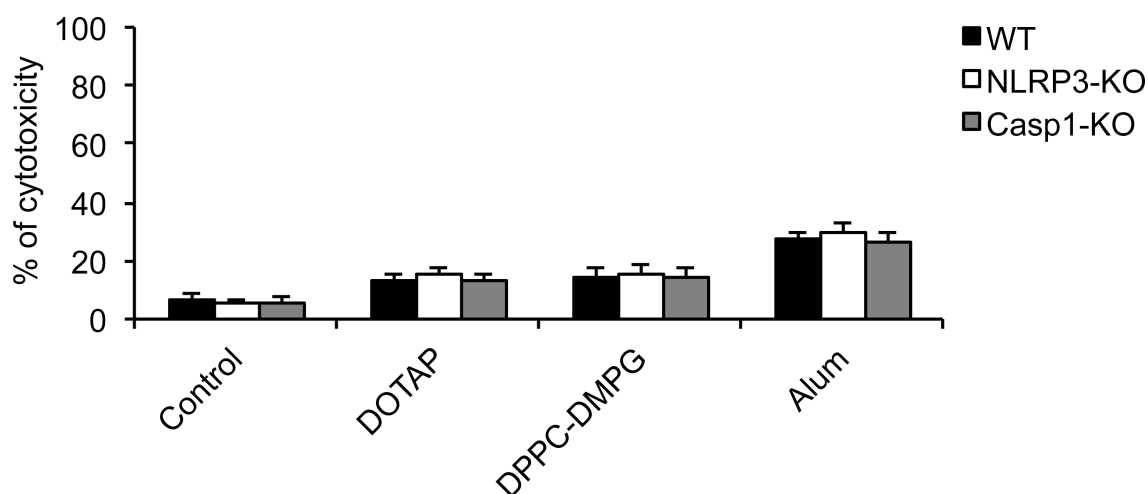


Figure 4.6: Macrophage viabilities after treatment with charged liposomes or alum. The levels of released Lactate dehydrogenase (LDH) were measured from the supernatants of LPS-primed immortalized wild-type, *Nlrp3*^{-/-} and *Caspase 1*^{-/-} macrophages that were stimulated with the DOTAP or DPPC-DMPG liposomes (70 µg/ml) or alum (500 µg/ml) for 6 h. Measurements were determined in triplicate and are shown as mean ± s.d., and data are from one of three independent experiments.

The intact NLRP3 inflammasome machinery is required for liposome-induced IL-1β secretion

Next, we sought to determine whether the intact NLRP3 inflammasome machinery was essential for liposome-induced IL-1β secretion. First, we observed that liposome-induced IL-1β secretion required LPS priming (**Figure 4.7**). Moreover, macrophages deficient in NLRP3, ASC or caspase-1, three key components of the NLRP3 inflammasome, all failed to respond to liposome stimulation after LPS priming (**Figure 4.8**). In contrast, macrophages deficient in NLRC4, another Nod-like receptor that recognizes bacterial flagellin (Miao et al., 2006), or AIM2 (absence in melanoma 2),

an immune sensor for DNA(Fernandes-Alnemri et al., 2009; Hornung et al., 2009), responded to charged liposomes stimulation in a manner similar to wild-type cells (**Figure 4.8** and **Figure 4.9**). These data collectively suggest that charged liposomes induced IL-1 β secretion specifically via activation of the NLRP3 inflammasome.

To further confirm that charged liposomes activate the NLRP3 inflammasome, we next tested whether potassium efflux, a seemingly obligatory event during NLRP3 inflammasome activation(Gross et al., 2011; Jin and Flavell, 2010; Ogura et al., 2006), was necessary for liposome-induced inflammasome activation. Indeed, the blockade of potassium efflux with a high concentration of KCl drastically decreased the liposome-induced NLRP3 inflammasome activation (**Figure 4.10**). Similar results were also obtained when glibenclamide, an inhibitor that blocks potassium channels(Dostert et al., 2009), was used (data not shown). Hereafter, we used DOTAP and DPPC-DMPG liposomes as the representative cationic and anionic liposomes, respectively, to investigate the mechanism by which charged liposomes activate the NLRP3 inflammasome.

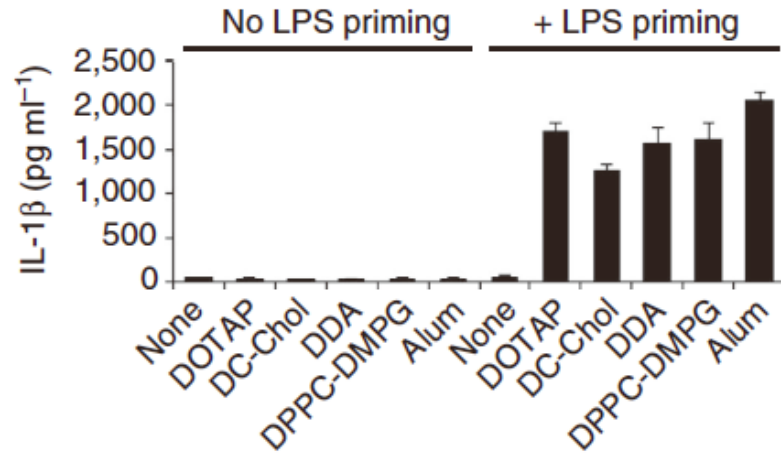


Figure 4.7: Liposomes-induced IL-1 β release requires LPS priming. ELISA for IL-1 β from the supernatants of LPS-primed or unprimed wild-type BMDMs that were stimulated with indicated liposomes (30 μ g/ml) or alum (250 μ g/ml). Data are shown as mean \pm s.d., and are representative of three independent experiments.

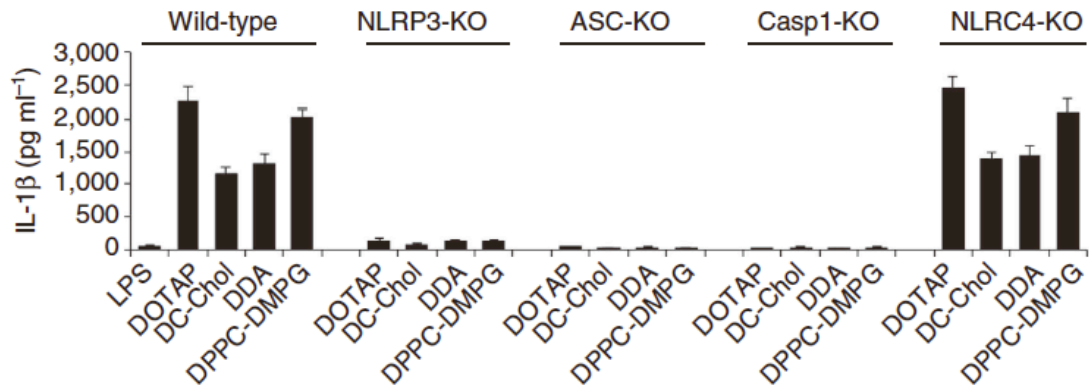


Figure 4.8: The NLRP3 inflammasome mediates liposomes-induced IL-1 β release. The IL-1 β levels from the supernatants of LPS-primed immortalized mouse macrophages from wild-type, *Nlrp3*^{-/-}, *Asc*^{-/-}, *Caspase-1*^{-/-} or *Nlr4*^{-/-} mice that were stimulated with liposomes (70 μ g/ml). Data are shown as mean \pm s.d., and are representative of three independent experiments.

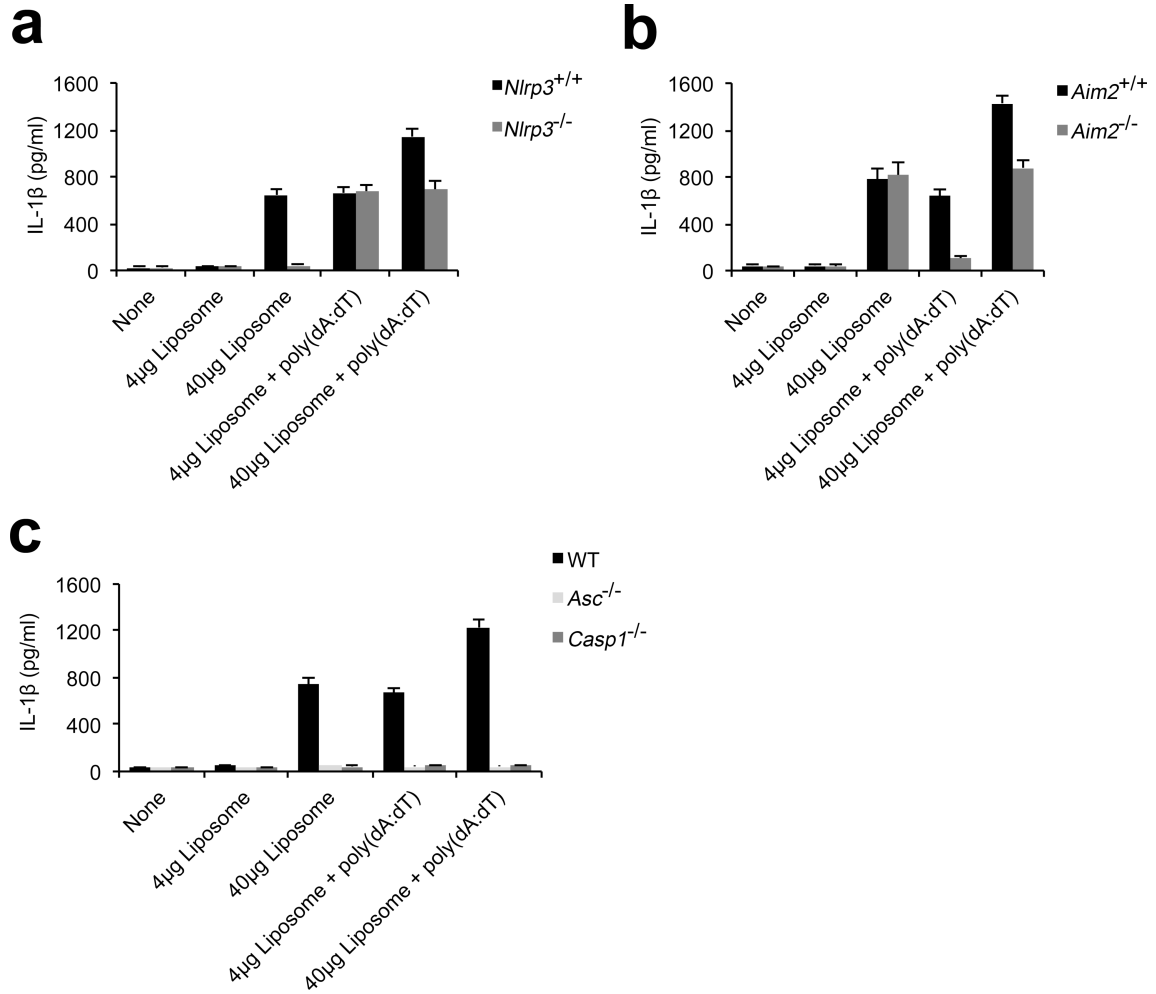


Figure 4.9: Charged liposomes activate the NLRP3, but not AIM2, inflammasome. (a-c) ELISA for IL-1 β from supernatants of LPS-primed immortalized murine macrophages from wild-type, *Nlrp3*^{-/-}, *Asc*^{-/-}, *Caspase 1*^{-/-} or *Aim2*^{-/-} mice that were stimulated with indicated amount of empty cationic liposomes (Lipofectamine 2000) or poly(dA:dT)-loaded liposomes for 6 h. Data are shown as mean \pm s.d., and are representative of three independent experiments.

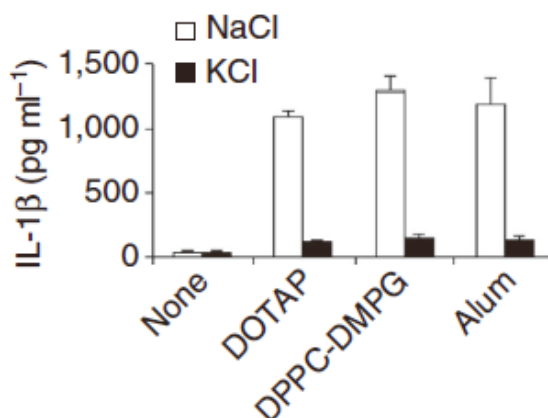


Figure 4.10: Potassium efflux is required for liposome-induced IL-1 β secretion. (a-c) ELISA for IL-1 β from the supernatants of LPS-primed wild-type BMDMs that were cultured in 150 mM of KCl or NaCl followed by stimulation with liposomes or alum. Data are shown as mean \pm s.d., and are representative of three independent experiments.

IL-1R signaling is critical for the adjuvant effect of liposomes

The mature bioactive IL-1 β is a powerful proinflammatory cytokine that is critically involved in both innate and adaptive immunities. It functions by signaling through its receptor, IL-1R. Since it has been previously suggested that IL-1 β -IL-1R signaling axis is crucial for directing Th2 responses (Shaw et al., 2011), we next tested whether the blockade of IL-1 β -IL-1R signaling pathway would affect the humoral immune response induced by antigen-encapsulated liposomes. As shown in **Figure 4.11a**, mice deficient in IL-1R had significantly reduced OVA-specific IgG1, the major subtype of antibodies, in comparison with their wild-type littermates. Notably, deficiency in IL-1R did not appear to affect the levels of OVA-specific IgGs when LPS was used as the adjuvant (**Figure 4.11b**). This suggests that IL-1R deficiency does not yield a

universal defect of mounting antibody responses. Together, these data indicate that IL-1 β -IL-1R signaling pathway is crucial for the adjuvanticity of liposomes.

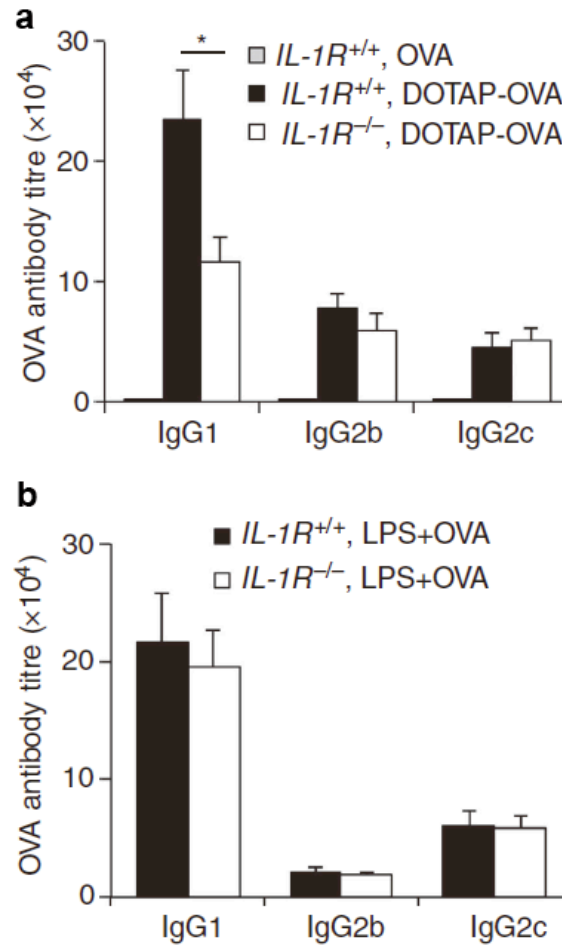


Figure 4.11: IL-1R signaling is crucial for liposomes' adjuvant effect. (a, b) Six- to eight-week old female wild-type (*Il1rl*^{+/+}) or *Il1rl*^{-/-} mice were subcutaneously immunized on day 0 and day 14 with 40 μ g/mouse ovalbumin (OVA) alone or the same amount of OVA encapsulated within DOTAP liposomes (a) or mixed with LPS (25 μ g/mouse, b). On day 24, the mice were sacrificed, and sera were collected and analyzed for OVA-specific IgG1, IgG2b, and IgG2c levels by ELISA. The data are shown as geometrical mean \pm s.e.m., and are representative of at least two independent experiments (n=4-5 mice per group). *, $p < 0.05$ versus controls. Statistical significance was determined by the standard two-tailed Student's *t*-test.

AIM2: DELINEATE THE MOLECULAR MECHANISM BY WHICH LIPOSOMES ACTIVATE THE NLRP3 INFLAMMASOME

Activation of the NLRP3 inflammasome requires cellular uptake of liposomes via the actin-dependent endocytic pathway

NLRP3 is a cytosolic innate immune receptor which, upon activation, recruits ASC and procaspase-1 to assemble into the inflammasome (Ogura et al., 2006). Therefore, we next tested whether cellular uptake of liposomes was necessary for activation of the NLRP3 inflammasome. The inhibition of the endocytic pathway with cytochalasin D, a potent inhibitor of actin polymerization (Hornung et al., 2008), greatly reduced liposome-induced IL-1 β secretion, whereas ATP-induced NLRP3 inflammasome activation, which does not require endocytic routes to activate the NLRP3 inflammasome (Hornung et al., 2008; Mariathasan et al., 2006), was largely unaffected (**Figure 4.12**). These data suggest that the cellular internalization of liposomes is an essential step for liposome-induced NLRP3 inflammasome activation.

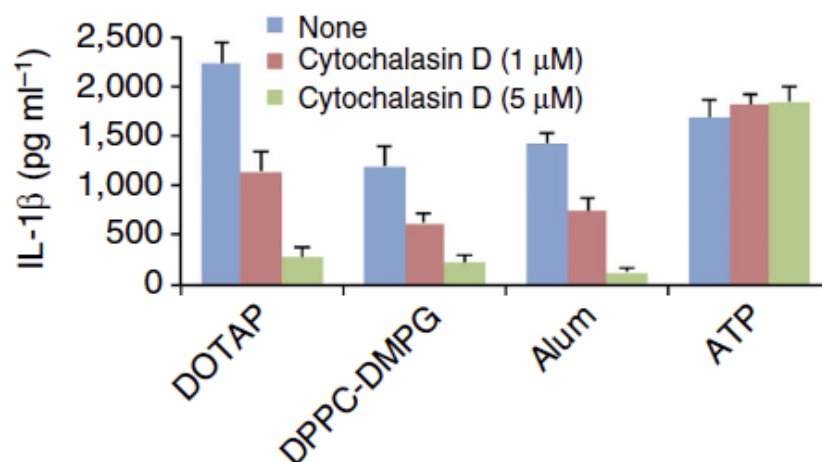


Figure 4.12: Activation of the NLRP3 inflammasome requires cellular uptake of liposomes. IL-1 β from the supernatants of PMA-primed THP1 cells that were pretreated with cytochalasin D before stimulation with indicated liposomes (DOTAP liposome 40 μ g/ml, DPPC-DMPG liposomes 100 μ g/ml), alum (500 μ g/ml) or ATP (2 mM). Data are shown as mean \pm s.d., and are representative of three independent experiments.

Lysosomal acidification and cathepsin B activity are dispensable for liposome-induced NLRP3 inflammasome activation

We also sought to determine whether the lysosome-cathepsin B pathway was involved in liposome-induced NLRP3 inflammasome activation. First, we found that the pharmacologic inhibition of lysosomal acidification, a process critical for crystal-induced inflammasome activation (Hornung et al., 2008), did not significantly affect liposome-induced NLRP3 inflammasome-mediated IL-1 β secretion (**Figure 4.13a**). Furthermore, we also observed that lysosomal cathepsin B was not necessary for liposome-triggered NLRP3 inflammasome activation because cathepsin B-deficient macrophages had only a minimal reduction in the amount of secreted IL-1 β after liposome stimulation (**Figure**

4.13b). Notably, we observed that liposome-induced IL-1 β secretion was decreased in BMDMs that were pretreated with a cathepsin B inhibitor, CA-074-Me (**Figure 4.13c**). These seemingly conflicting data may be due to the potential off-target side effects of CA-074-Me (Dostert et al., 2009; Hornung et al., 2008; Newman et al., 2009). Taken together, these data indicate that liposomes may either activate the NLRP3 inflammasome in a lysosome-independent manner or that several lysosomal cathepsins, such as cathepsins B and L, are functionally redundant in mediating liposome-induced NLRP3 inflammasome activation.

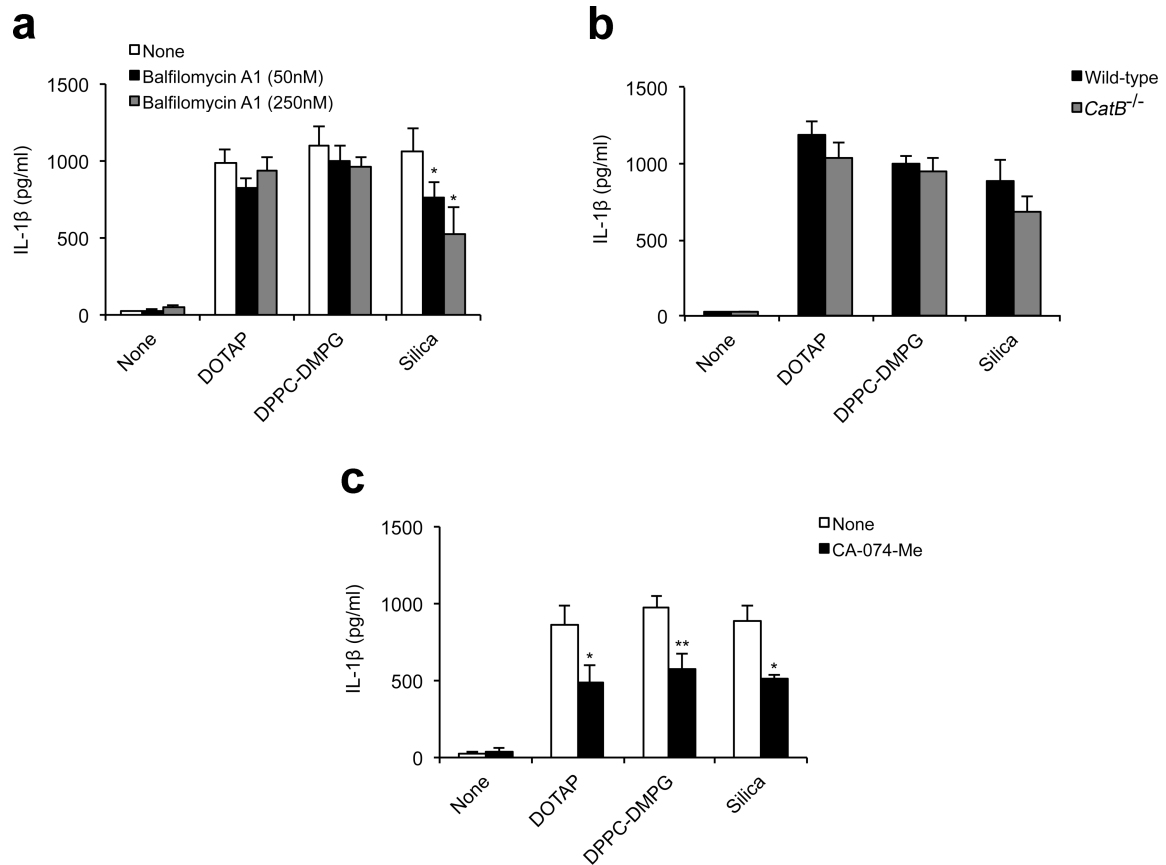


Figure 4.13: Lysosomal cathepsin-B is dispensable for liposome-induced NLRP3 inflammasome activation. (a, c) ELISA for IL-1 β from supernatants of LPS-primed wild-type BMDMs that were pretreated with bafilomycin A1 (50 nM and 250 nM in a) or CA-074-Me (10 μ M in c) followed by stimulations with DOTAP or DPPC-DMPG liposomes (30 μ g/ml) or silica (500 μ g/ml). (b) ELISA for IL-1 β from supernatants of LPS-primed immortalized murine wild-type or cathepsin B deficient (*CatB*^{-/-}) macrophages that were stimulated with DOTAP or DPPC-DMPG liposomes (70 μ g/ml). Data are shown as mean \pm s.d., and all data are representative of at least three independent experiments. *, $p < 0.05$, and **, $p < 0.01$ versus controls. Statistical significance was determined by the standard two-tailed Student's *t*-test.

Charged liposomes, but not neutral ones, trigger the production of ROS production from mitochondria to direct NLRP3 inflammasome activation

Although mitochondrial ROS are essential for activation of the NLRP3 inflammasome (Kepp et al., 2011; Nakahira et al., 2011; Tschopp, 2011; Zhou et al., 2011), a recent study also suggested a role for ROS during the first priming step of pro-IL-1 β synthesis (Bauernfeind et al., 2011). Therefore, to specifically investigate the role of ROS during activation of the NLRP3 inflammasome activation but not during pro-IL-1 β synthesis, we added ROS inhibitors after the synthesis of pro-IL-1 β was completed. This was achieved through prolonged LPS stimulation to saturate NF- κ B activation before ROS inhibition. As shown in **Figure 4.14**, prolonged LPS priming for 18 h maximized the level of NF- κ B mediated transcription, which was measured by the production of NF- κ B-dependent TNF.

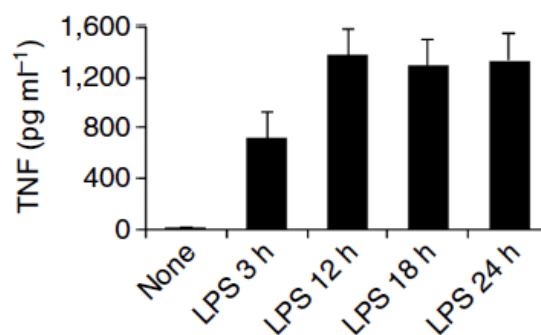


Figure 4.14: Prolonged LPS stimulation saturates LPS-induced NF- κ B activation. ELISA for TNF from the supernatants of wild-type BMDMs that were stimulated with 100 ng/ml LPS for various periods of time as indicated. Data are shown as mean \pm s.d., and are representative of at least three independent experiments.

We then evaluated whether different liposomes could induce mitochondrial ROS production. Notably, only the charged liposomes, but not neutral liposomes, induced the robust generation of mitochondrial ROS in LPS-primed macrophages (**Figure 4.15**). Moreover, even in the absence of an LPS priming step, charged liposomes remained capable of increasing the mitochondrial ROS levels, suggesting that LPS pre-stimulation is not necessary for mitochondrial ROS induction (data not shown). Next, we used the pharmacological inhibitor diphenyleneiodonium (DPI), which blocks mitochondrial ROS production when used at high concentrations (Bulua et al., 2011; Holland and Sherratt, 1972), to further investigate whether mitochondrial ROS were involved in liposome-induced NLRP3 inflammasome activation. The addition of DPI prior to liposome stimulation drastically reduced liposome-induced mitochondrial ROS production and IL-1 β release (**Figure 4.16**). Notably, the reduction in IL-1 β secretion was not due to the inhibition of NF- κ B activation by DPI because neither the levels of pro-IL-1 β or Nlrp3 were significantly changed after the 18 h LPS priming step (**Figure 4.17**). In contrast to DPI, pretreatment of the macrophage with VAS2870, which blocks NADPH oxidase (NOX)-generated ROS (Stielow et al., 2006), did not affect liposome-induced IL-1 β secretion, suggesting that mitochondria, rather than NOX-derived ROS drive the NLRP3 inflammasome activation (**Figure 4.18**). Moreover, because activation of the AIM2 inflammasome by poly(dA:dT) was not affected by DPI treatment (**Figure 4.16b**), the inhibition of mitochondrial ROS seemed to specifically affect the NLRP3 inflammasome. Similar results were also observed when other ROS inhibitors, such as (2R, 4R)-4-aminopyrrolidine-2,4-dicarboxylate (APDC) or N-acetyl-cysteine (NAC), were used

(data not shown). Taken together, these data indicate that mitochondrial ROS production is essential for NLRP3 inflammasome activation by charged liposomes and that the failure of neutral liposomes to activate the NLRP3 inflammasome directly correlates with their inability to induce mitochondrial ROS.

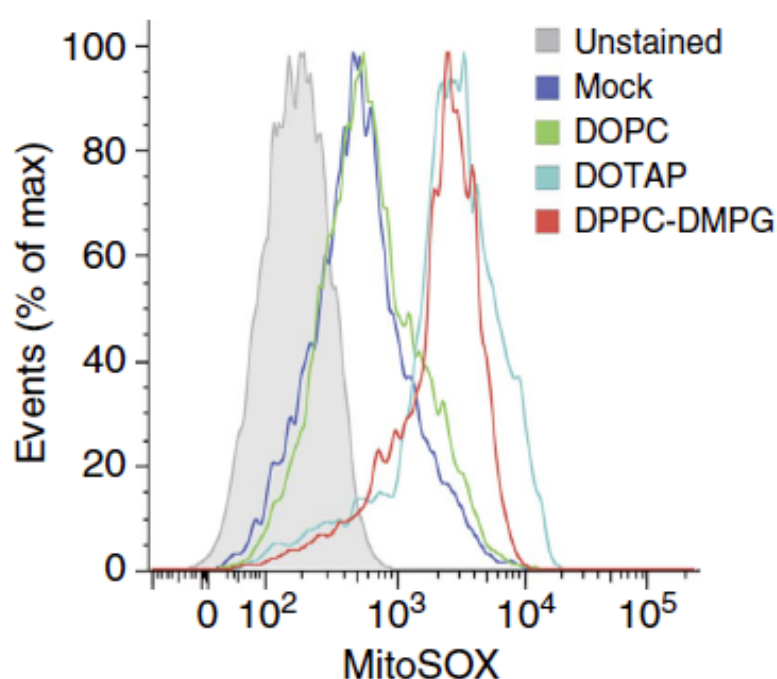


Figure 4.15: Charged but not neutral liposomes induce mitochondrial ROS production. Mitochondrial ROS production was measured by flow cytometry in LPS-primed BMDMs that were stimulated with indicated liposomes (30 $\mu\text{g}/\text{ml}$) and labeled with MitoSOX. Data are shown as mean \pm s.d., and are representative of at least three independent experiments.

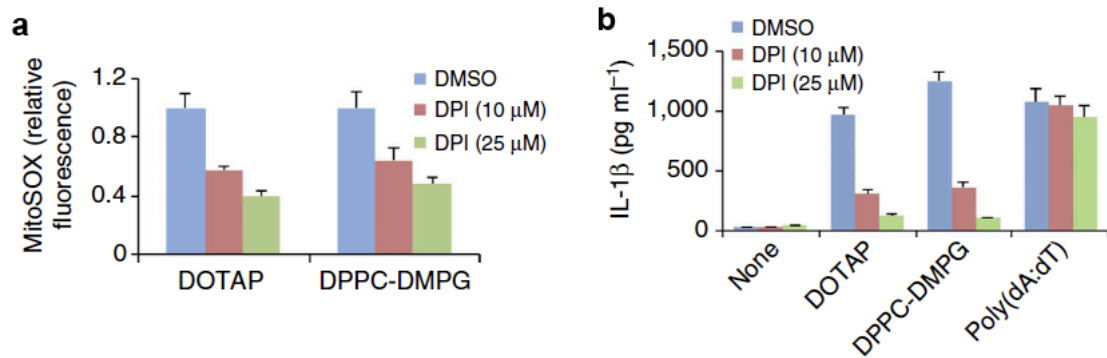


Figure 4.16: Inhibition of mitochondrial ROS abolishes liposome-induced inflammasome activation. (a) LPS-primed wild-type BMDMs were pretreated with DPI for 30 min and then stimulated with indicated liposomes (30 μ g/ml) for 6 h. The cells were then stained with MitoSOX and the levels of mitochondrial ROS were normalized to the untreated controls (n=3). (b) ELISA for IL-1 β from the supernatants of LPS-primed (18 h) BMDMs that were pretreated with DPI before stimulation with indicated liposomes (30 μ g/ml) or poly(dA:dT) (2 μ g/ml). Data are shown as mean \pm s.d., and the data are representative of three (a) and four (b) independent experiments.

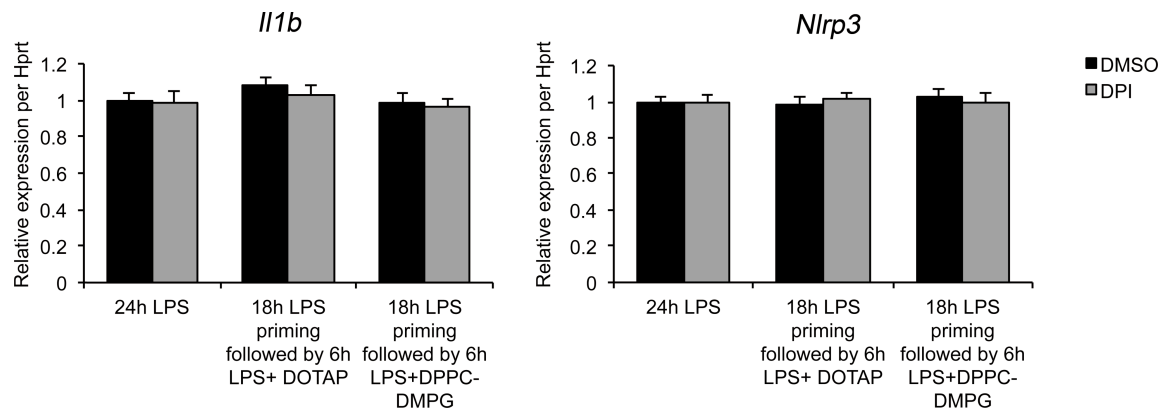


Figure 4.17: DPI treatment does not affect *Il1b* and *Nlrp3* mRNAs after prolonged LPS priming. The mRNA levels of *Il1b* (left) and *Nlrp3* (right) were quantified by real-time RT-PCR in wild-type BMDMs that were stimulated as indicated. DPI (25 μ M) was added after 18 h LPS priming but 45min before addition of liposomes. Gene expression data are presented as relative expression to HPRT1, and the gene relative expression level in 24 h LPS treated wild-type macrophages (DMSO group) were designated as 1. Data are representative of three independent experiments.

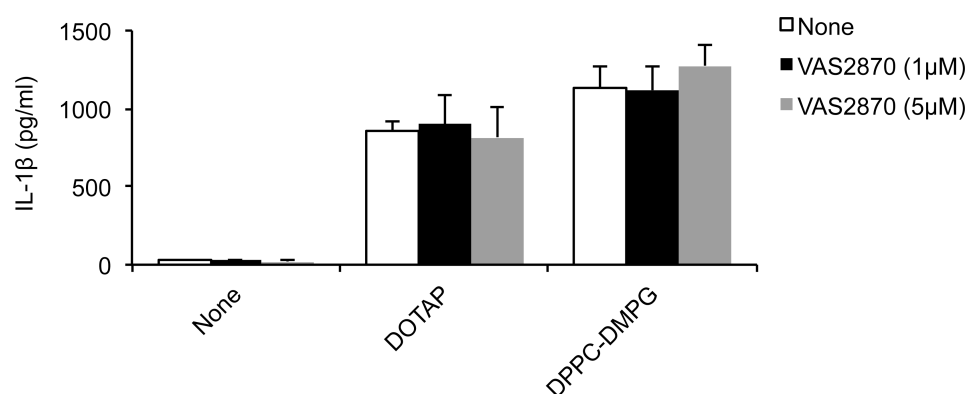


Figure 4.18: Inhibition of NADPH oxidase does not affect liposome-induced IL-1 β secretion. The levels of IL-1 β were measured by ELISA from supernatants of LPS-primed wild-type BMDMs that were pretreated with VAS2870 for 30 min followed by stimulations with DOTAP or DPPC-DMPG liposomes (30 μ g/ml) for 6 h. Data are shown as mean \pm s.d., and are representative of three independent experiments.

AIM3: DELINEATE WHETHER TRPM2 IS CRITICAL FOR ACTIVATION OF THE NLRP3 INFLAMMASOME IN RESPONSE TO PARTICLES STIMULATION

Particulates (e.g. liposomes and crystals) induce a ROS-dependent Ca²⁺ influx via the TRPM2 channel

Our findings indicated that, similar to crystals (Tschopp and Schroder, 2010), liposomes activate the NLRP3 inflammasome via the induction of mitochondrial ROS. However, little is known about the mechanism by which ROS promote NLRP3 inflammasome activation. Due to the existence of a diverse pool of stimuli (Gross et al., 2011), these agonists may activate a common signaling intermediate that directs assembly of the NLRP3 inflammasome. Recently, it has been shown that accumulation of ROS can

induce a calcium influx via the TRPM2 (transient receptor potential melastatin 2) channel (Hecquet and Malik, 2009; Yamamoto et al., 2008). TRPM2 is expressed by immune cells, such as dendritic cells, monocytes, macrophages, PMNs and lymphocytes, and is a calcium-permeable nonselective cation channel that plays a crucial role in innate immune regulation (Sumoza-Toledo and Penner, 2011). TRPM2-mediated calcium influx has been implicated in ROS-induced chemokine production in monocytes (Yamamoto et al., 2008). Notably, TRPM2-deficient mice are also susceptible to infection of *Listeria monocytogenes* (Knowles et al., 2011), a known activator of the NLRP3 inflammasome (Kim et al., 2010; Mariathasan et al., 2006). Because calcium appears to be a key mediator of various signaling events under stressed conditions, we hypothesize that liposomes/crystals induce ROS-dependent TRPM2-mediated calcium influx, which eventually signals assembly and activation of the NLRP3 inflammasome.

As shown in **Figure 4.19**, we first observed that charged liposomes or crystals induced the increase in the concentration of intracellular free calcium ($[Ca^{2+}]_i$). This elevation in $[Ca^{2+}]_i$ was due to calcium influx across the plasma membrane because the removal of extracellular calcium from the buffer solution dramatically inhibited the increase of $[Ca^{2+}]_i$ (**Figure 4.20** and **Figure 4.21**). Furthermore, blockade of mitochondrial ROS by DPI also prevented liposome/crystal-induced calcium influx, suggesting that calcium influx is ROS-dependent. Notably, BMDMs deficient in TRPM2 had similar defects in calcium influx after liposome/crystal stimulation, indicating that calcium influx is mediated by the TRPM2 channel (**Figure 4.20** and **Figure 4.21**). To further verify the role of TRPM2 in mediating calcium influx, we tested whether

inhibition of TRPM2 activation affected liposome/crystal-induced calcium influx. TRPM2 channel can be gated by ADP-ribose, a metabolic product formed during cellular exposure to ROS(Knowles et al., 2011; Sumoza-Toledo and Penner, 2011). Therefore, we treated LPS-primed wild-type BMDMs with DPQ, an inhibitor of poly(ADP-ribose) polymerase, to prevent ADP-ribose accumulation(Fonfria et al., 2004; Suto et al., 1991) before the addition of liposomes/crystals to determine whether the calcium influx was affected. As expected, DPQ significantly reduced calcium influx in response to stimulation with particles (**Figure 4.20** and **Figure 4.21**). These data collectively indicate that stimulation of macrophages with either liposomes or crystals can trigger ROS-dependent TRPM2-mediated calcium influx.

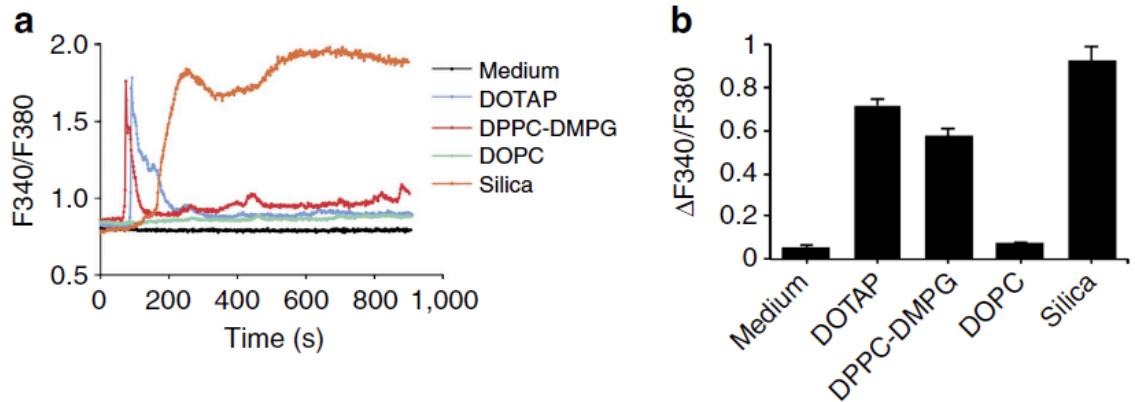


Figure 4.19: Charged liposomes and crystals induce cytosolic calcium elevation. (a) The change in $[Ca^{2+}]_i$ over time was represented by the fluorescence of Fura-2 at 340 nm to that at 380 nm (F340/F380) and (b) the maximum $[Ca^{2+}]_i$ elevations, represented by $\Delta F340/F380$, are shown in LPS-primed wild-type BMDMs treated with medium alone, cationic liposomes (DOTAP, 30 $\mu\text{g/ml}$), neutral liposomes (DOPC, 30 $\mu\text{g/ml}$), anionic liposomes (DPPC-DMPG, 30 $\mu\text{g/ml}$) or silica crystals (300 $\mu\text{g/ml}$). The data are representative of at least three independent experiments and are shown as mean \pm s.e.m. in **b** (n=22-31).

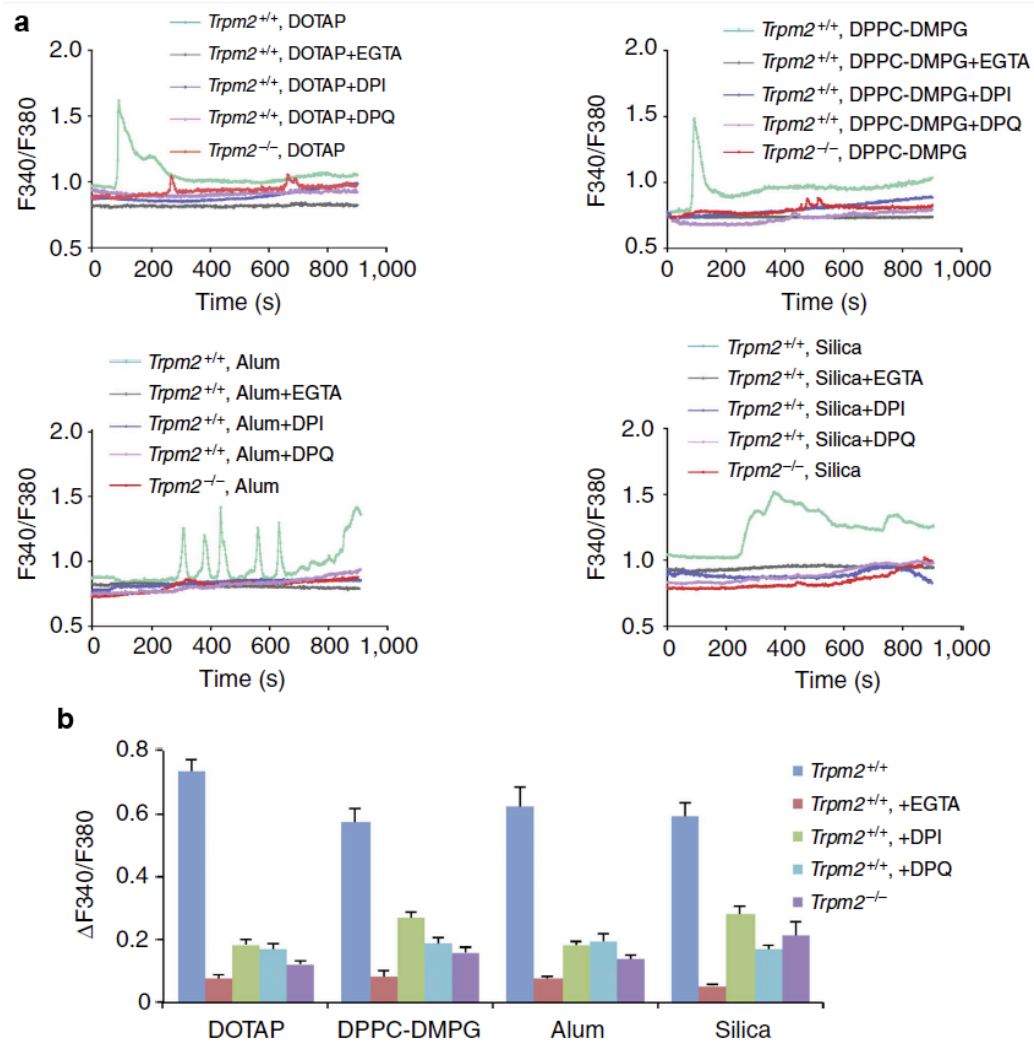


Figure 4.20: Charged liposomes and crystals induce a ROS-dependent TRPM2-mediated calcium influx. (a) The time-dependent change in $[Ca^{2+}]_i$, represented by F340/F380, in the LPS-primed *Trpm2*^{+/+} BMDMs cultured in calcium-containing solution that were pretreated with DPI (25 μ M) or DPQ (200 μ M) for 45min before the addition of the indicated liposomes (30 μ g/ml) or crystals (alum, 400 μ g/ml; silica, 300 μ g/ml) is shown. The same doses of liposomes or crystals were also used to stimulate the LPS-primed *Trpm2*^{-/-} BMDMs cultured in calcium-containing solution. The $[Ca^{2+}]_i$ change over time, represented by F340/F380, after stimulation with liposomes or crystals in LPS-primed *Trpm2*^{+/+} BMDMs cultured in calcium-free 0.5 mM EGTA-containing solution is also shown. Inflammasome agonists were added 1 min after the initiation of calcium recording as shown in **a**. (b) The maximum $[Ca^{2+}]_i$ elevations, represented by $\Delta F340/F380$, are shown for LPS-primed BMDMs in response to the stimulations described in **a**. The data are representative of at least three independent experiments and are shown as mean \pm s.e.m. in **b** (n=21-34).

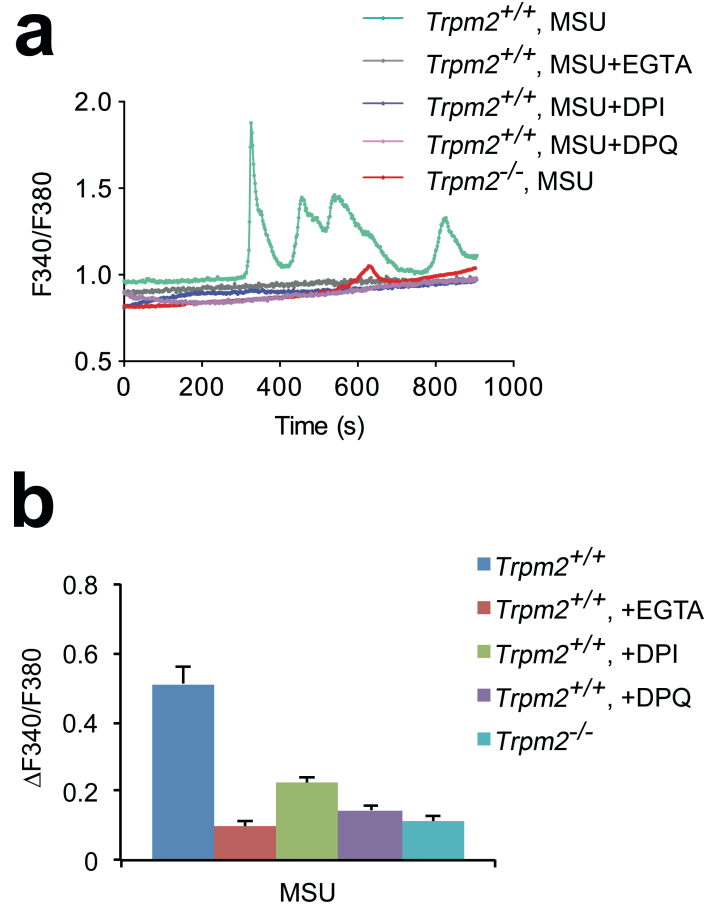


Figure 4.21: MSU crystals induce a TRPM2-mediated Ca^{2+} influx in macrophages. (a) Time-dependent change of $[Ca^{2+}]_i$, represented by F340/F380, was shown in LPS-primed $Trpm2^{+/+}$ BMDMs cultured in calcium-containing solution that were pretreated with DPI (25 μ M) or DPQ (200 μ M) for 45min for before the addition of MSU crystals (250 μ g/ml). The same doses of MSU crystals were used to stimulate the LPS-primed $Trpm2^{-/-}$ BMDMs cultured in calcium-containing solution. The $[Ca^{2+}]_i$ over time after additions of these stimuli into LPS-primed $Trpm2^{+/+}$ BMDMs cultured in calcium-free 0.5 mM EGTA-containing solution are also shown. MSU crystals were added 1 min after the starting of calcium recording. (b) The maximum $[Ca^{2+}]_i$ elevations, represented by $\Delta F340/F380$, were shown in LPS-primed BMDMs in response to the stimulations described in a. Data are representative of two independent experiments and are shown as mean \pm s.e.m. in b (n=21-38).

TRPM2-mediated Ca^{2+} influx regulates particle-induced IL-1 β secretion

We next determined whether TRPM2-mediated calcium influx was critical for IL-1 β release. *Trpm2*^{-/-} BMDMs demonstrated significantly reduced IL-1 β secretion in responses to stimulation with the liposomes DOTAP and DPPC-DMPG or the crystals of alum, silica and MSU. In contrast, poly(dA:dT)-induced IL-1 β secretion, which is AIM2 inflammasome-dependent (Fernandes-Alnemri et al., 2009; Hornung et al., 2009), was largely unaffected (Figure 4.22). Similarly, the removal of extracellular calcium from the culture medium significantly impaired the particulate agonist-, but not poly(dA:dT)-, induced IL-1 β release (Figure 4.22).

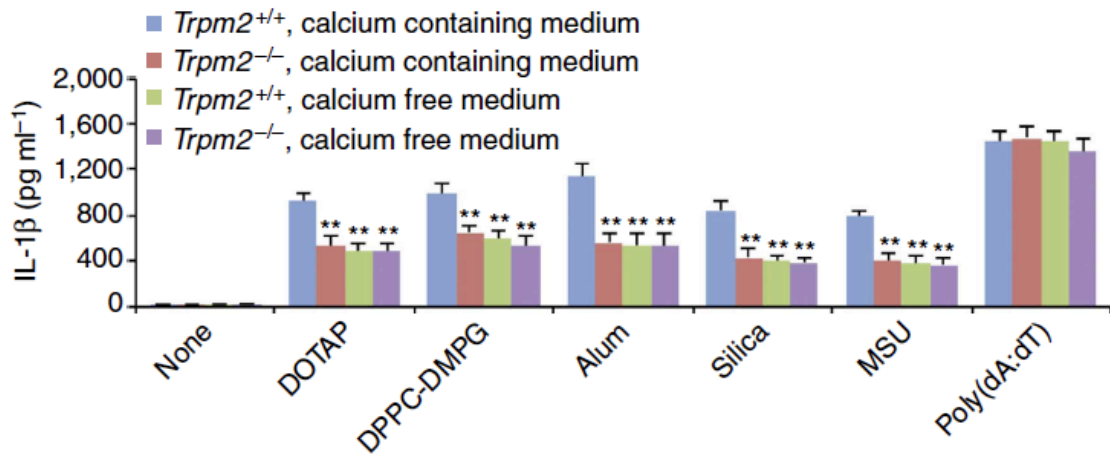


Figure 4.22: TRPM2-mediated Ca^{2+} influx regulates IL-1 β secretion. ELISA for IL-1 β from the supernatants of LPS-primed wild-type (*Trpm2*^{+/+}) or *Trpm2*^{-/-} BMDMs that were stimulated with indicated liposomes (30 $\mu\text{g/ml}$), crystals (alum, 250 $\mu\text{g/ml}$; silica and MSU crystals, 200 $\mu\text{g/ml}$) or poly(dA:dT) (2 $\mu\text{g/ml}$) for 6 h in calcium-containing or calcium-free medium. All data are representative of at least three independent experiments, and are shown as mean \pm s.d.. *, $p < 0.05$, and **, $p < 0.01$ versus controls. Statistical significance was determined by the standard two-tailed Student's *t*-test.

Furthermore, we observed that the decrease in the mature IL-1 β secretion in Trpm2 $^{-/-}$ BMDMs was not due to the reduction in either pro-IL-1 β or NLRP3 levels, the genes for which are regulated by NF- κ B (Bauernfeind et al., 2009; Gross et al., 2011) because TRPM2 deficiency affected neither pro-IL-1 β nor NLRP3 (**Figure 4.23** and data not shown). Consistent with this finding, TNF secretion was not affected by TRPM2 deficiency (**Figure 4.24**). Moreover, the inhibition of TRPM2 activation by DPQ also significantly impaired liposome-/crystal-, but not poly(dA:dT)-, induced IL-1 β secretion (**Figure 4.25**). In contrast to the wild-type cells, Trpm2 $^{-/-}$ BMDMs did not display any further reduction in IL-1 β secretion after DPQ treatment (**Figure 4.26**), indicating that DPQ treatment specifically blocked TRPM2-mediated IL-1 β release. It has been shown that the inhibition of key enzymes in the mitochondrial respiratory chain can artificially increase the levels of mitochondrial ROS and trigger spontaneous NLRP3 inflammasome activation in the absence of inflammasome stimuli (Zhou et al., 2011), therefore we next verified the role of TRPM2 in mediating IL-1 β release under these conditions. To do so, we artificially induced the accumulation of mitochondrial ROS with antimycin A, an inhibitor that specifically blocks the respiratory chain complex III, and determined whether TRPM2 deficiency affected antimycin A-induced IL-1 β release. Indeed, we found that Trpm2 $^{-/-}$ BMDMs had significantly reduced secretion of IL-1 β , but not TNF (**Figure 4.27**). Consistently, TRPM2 deficiency also decreased IL-1 β secretion in response to direct stimulation with ROS, such as H₂O₂ (**Figure 4.27**). Together, these results suggest a critical role for TRPM2-mediated calcium influx in bioactive IL-1 β

release, and demonstrate that TRPM2 deficiency specifically impairs the processing of immature pro-IL-1 β into its mature form.

It should be noted that in addition to TRPM2-dependent IL-1 β release, liposomes/crystals also induced TRPM2-independent IL-1 β secretion. This effect was most likely mediated by ER-calcium release via IP3R, as suggested in a recent study by Murakami et al.(Murakami et al., 2012). In support of the hypothesis that calcium influx and ER calcium release are both essential for NLRP3 inflammasome activation, we observed that the intracellular calcium chelator BAPTA-AM, which blocks [Ca²⁺]_i elevation regardless of the source of calcium, almost completely inhibited IL-1 β release (**Figure 4.28**) whereas the inhibition of either calcium influx or intracellular ER calcium release(Murakami et al., 2012) only partially reduced IL-1 β secretion. Together, these results demonstrate that both calcium influx and ER calcium release are critical for activation of the NLRP3 inflammasome.

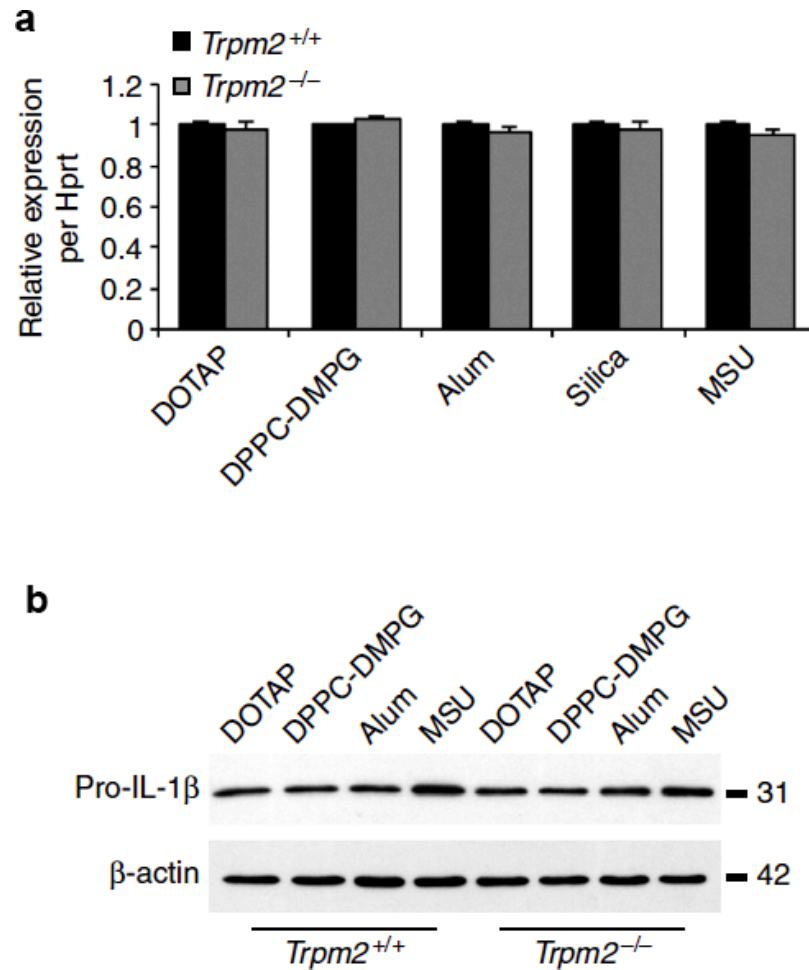


Figure 4.23: TRPM2 deficiency does not affect the levels of pro-IL-1 β . (a) The levels of pro-IL-1 β mRNA were quantified by real-time RT-PCR in LPS-primed *Trpm2*^{+/+} or *Trpm2*^{-/-} BMDMs after stimulation with indicated liposomes and crystals. The gene expression data are presented as expression relative to HPRT1, and the relative gene expression levels in wild-type macrophages were designated as 1. (b) Immunoblots for pro-IL-1 β and β -actin in the cell lysates from LPS-primed *Trpm2*^{+/+} or *Trpm2*^{-/-} BMDMs after stimulation with liposomes or crystals. All data are representative of at least three independent experiments, and are shown as mean \pm s.d. in a.

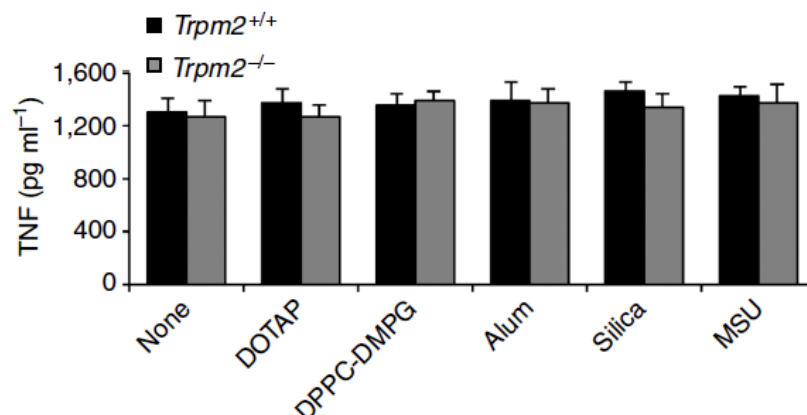


Figure 4.24: TRPM2 deficiency does not affect TNF secretion. ELISA TNF from the supernatants of LPS-primed wild-type (*Trpm2*^{+/+}) or *Trpm2*^{-/-} BMDMs that were stimulated with indicated liposomes (30 μ g/ml) or crystals (alum, 250 μ g/ml; silica and MSU crystals, 200 μ g/ml) for 6 h in calcium-containing medium. All data are representative of at least three independent experiments, and are shown as mean \pm s.d..

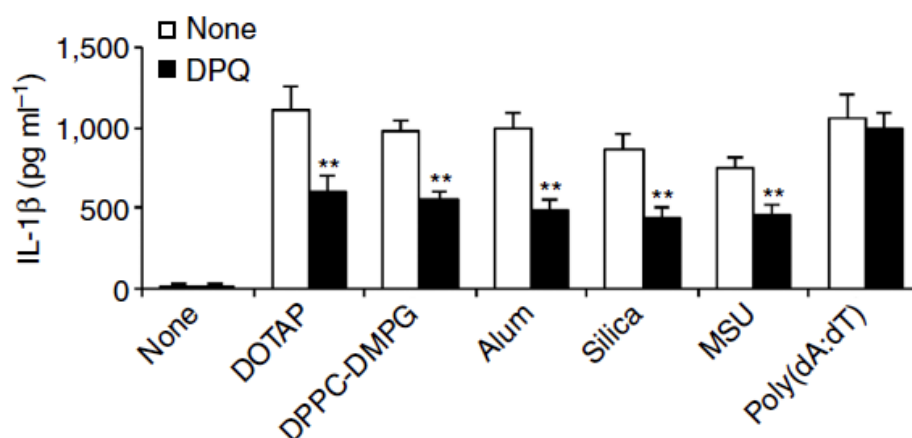


Figure 4.25: Inhibition of TRPM2 by DPQ significantly impairs liposome-induced IL-1 β secretion. IL-1 β from the supernatants of LPS-primed wild-type BMDMs that were pretreated with DPQ (200 μ M) followed by inflammasome agonist stimulation. All data are representative of at least three independent experiments, and are shown as mean \pm s.d..

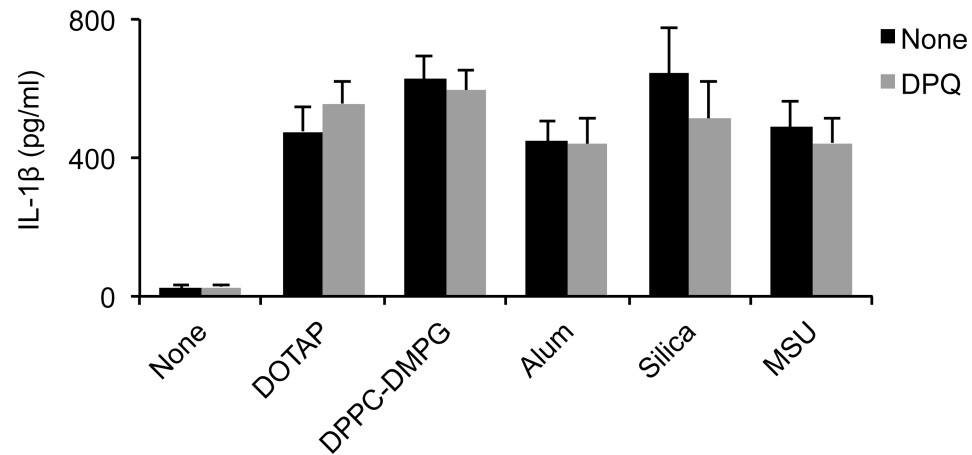


Figure 4.26: DPQ does not further reduce particles-induced IL-1 β release in *Trpm2*^{-/-} BMDMs. ELISA for IL-1 β from supernatants of LPS-primed *Trpm2*^{-/-} BMDMs that were pretreated with DPQ (200 μ M) followed by stimulations with indicated liposomes or crystals. Data are shown as mean \pm s.d., and are representative of three independent experiments.

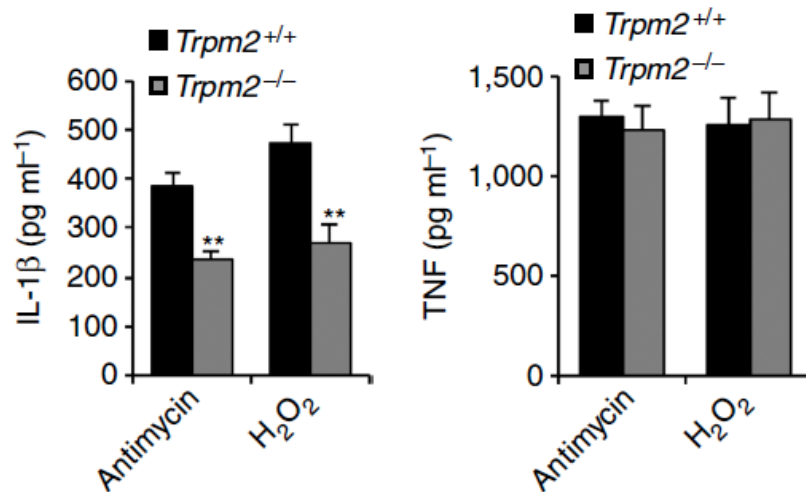


Figure 4.27: TRPM2 deficiency impairs ROS-induced IL-1 β secretion . IL-1 β (left) or TNF (right) from the supernatants of LPS-primed wild-type BMDMs that were stimulated with antimycin A (20 μ g/ml) or H₂O₂ (10 mM) for 6 h. All data are representative of at least three independent experiments, and are shown as mean \pm s.d.. *, $p < 0.05$, and **, $p < 0.01$ versus controls. Statistical significance was determined by the standard two-tailed Student's *t*-test.

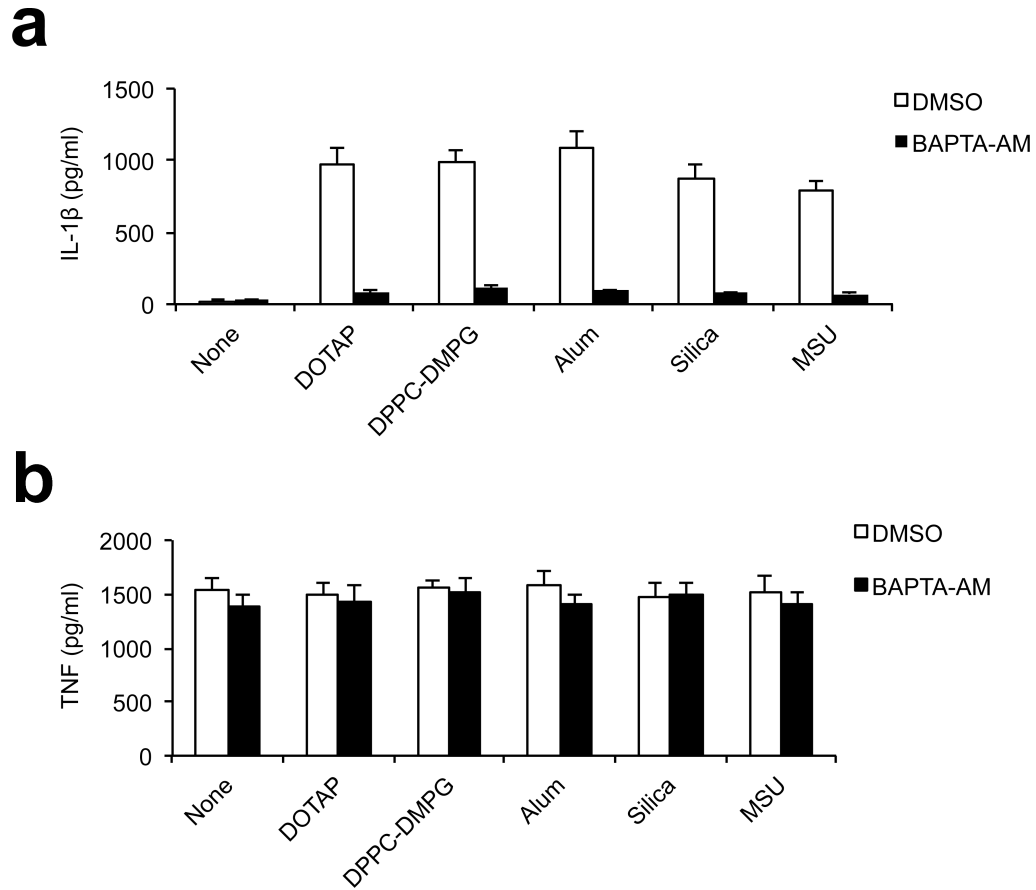


Figure 4.28: Blocking $[Ca^{2+}]_i$ elevation abolishes the NLRP3 inflammasome activation by particulates. ELISA for IL-1 β (a) or TNF (b) from supernatants of LPS-primed wild-type BMDMs that were pretreated with BAPTA-AM (25 μ M) followed by stimulations with liposomes or crystals. Data are shown as mean \pm s.d., and are representative of three independent experiments.

TRPM2 is critical for particle-induced, NLRP3-mediated caspase-1 activation

To determine the mechanism by which TRPM2 deficiency leads to impaired processing of bioactive IL-1 β , we tested whether *Trpm2*^{-/-} BMDMs were defective in caspase-1 activation. As shown in **Figure 4.29**, deficiency in TRPM2 or the blockade of $[Ca^{2+}]_i$ increase via BAPTA-AM drastically reduced the level of mature caspase-1 in

response to liposomes or crystals. This defect was not due to impaired mitochondrial ROS production because neither genetic ablation of TRPM2 nor removal of extracellular calcium impaired mitochondrial ROS production after liposome stimulation (**Figure 4.30** and **Figure 4.31**). Moreover, we found that *Nlrp3*^{-/-} macrophages displayed a comparable level of calcium influx as wild-type cells in response to liposome stimulation (**Figure 4.32**), suggesting that TRPM2-mediated calcium influx is an early event before assembly of the NLRP3 inflammasome. Together, these data indicate that ROS-dependent calcium influx via the TRPM2 channel is critical for NLRP3 inflammasome-mediated caspase-1 activation.

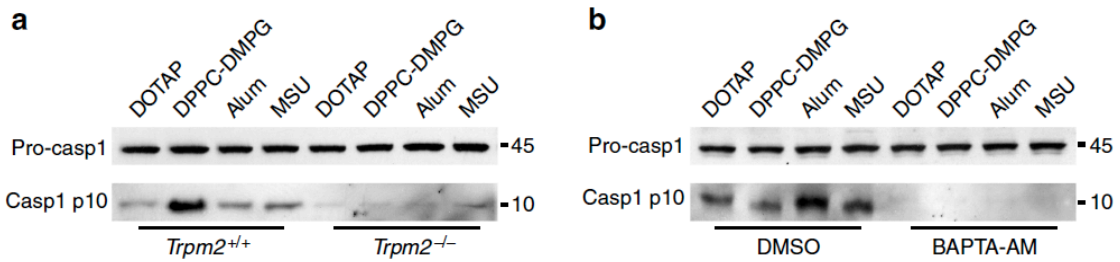


Figure 4.29: Blocking $[Ca^{2+}]_i$ elevation or inhibiting TRPM2 impairs caspase-1 activation by particulates. (a) Immunoblots of procaspase-1 in lysates and caspase-1 p10 in culture supernatants of LPS-primed *Trpm2*^{+/+} and *Trpm2*^{-/-} BMDMs that were stimulated with indicated liposomes (30 μ g/ml) or crystals (250 μ g/ml). (b) Immunoblots of procaspase-1 and caspase-1 p10 in LPS-primed wild-type BMDMs that were pretreated with either BAPTA-AM (25 μ M) or DMSO before stimulation with the indicated liposomes (50 μ g/ml) and crystals (400 μ g/ml). One of three independent experiments is shown in **a** and **b**.

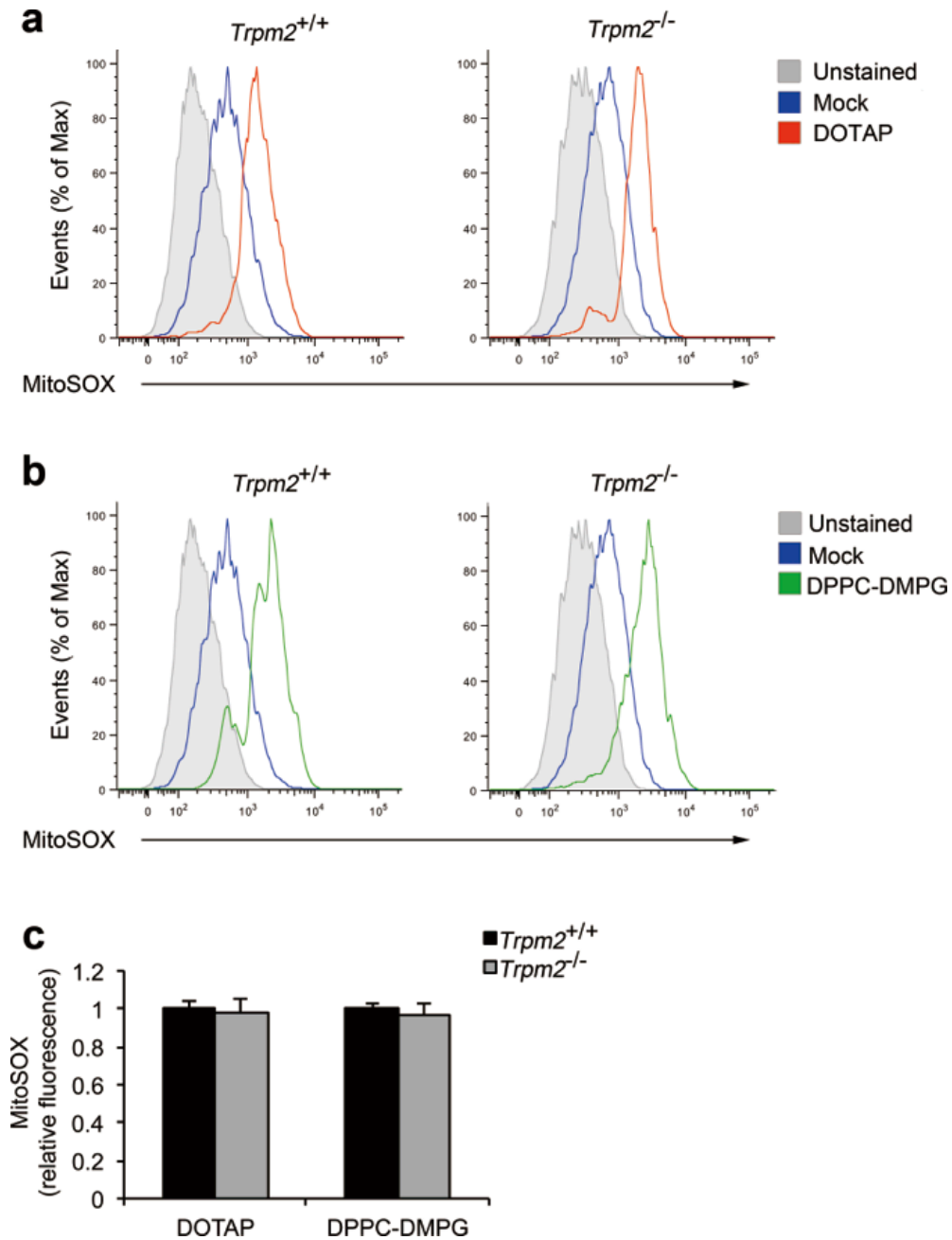


Figure 4.30: TRPM2 deficiency does not affect liposome-induced mitochondrial ROS production. (a-c) LPS-primed *Trpm2*^{+/+} or *Trpm2*^{-/-} BMDMs were treated with indicated liposomes (30 μ g/ml) for 6 h. The cells were then stained with MitoSOX and the levels of mitochondrial ROS were measured by flow cytometry (a, b). The data are representative for three experiments and are normalized to the untreated controls (c, n=3).

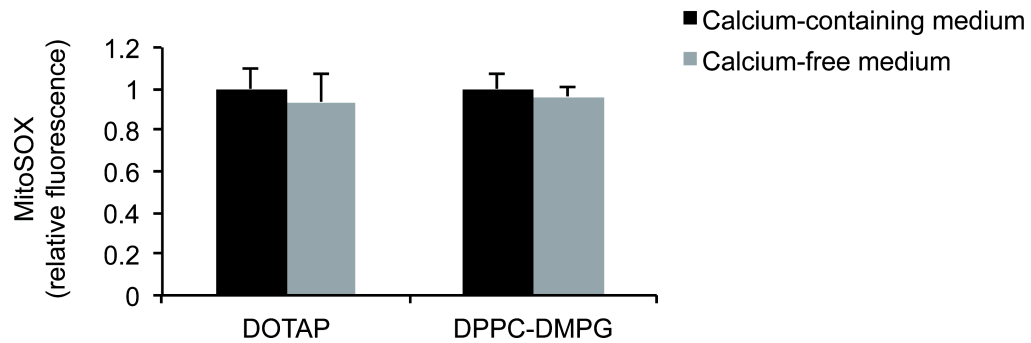


Figure 4.31: Removal of extracellular calcium does not impair mitochondrial ROS production. LPS-primed wild-type BMDMs were treated with indicated liposomes (30 $\mu\text{g}/\text{ml}$) for 6 h in the presence of calcium containing or calcium free medium. Then the cells were stained with MitoSOX and the levels mitochondrial ROS were measured by flow cytometry. Data are representative for two independent experiments and are normalized to untreated controls ($n=3$).

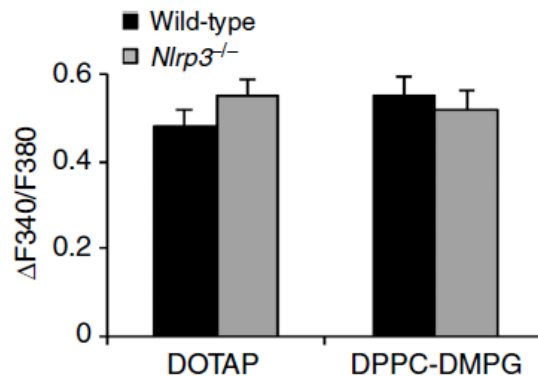


Figure 4.32: Calcium influx is an early event before the assembly of NLRP3 inflammasome. The maximum $[\text{Ca}^{2+}]_i$ elevations, represented by $\Delta F340/F380$, are shown for LPS-primed immortalized murine wild-type or *Nlrp3*^{-/-} macrophages in response to stimulation with liposomes (70 $\mu\text{g}/\text{ml}$). The data are shown as mean \pm s.e.m. ($n=23-28$), and are representative of two independent experiments.

Along with the role of calcium influx in activation of the NLRP3 inflammasome, potassium efflux has been shown to be essential for inflammasome activation by most, if

not all, NLRP3 inflammasome agonists (Jin and Flavell, 2010; Tschopp and Schroder, 2010). It appears that the mobilization of both cations across the plasma membrane is crucial for NLRP3 inflammasome activation. Consistent with a recent study (Gross et al., 2012), we found that the blockade of potassium efflux did not prevent calcium influx induced by NLRP3 inflammasome agonists (**Figure 4.33**), which further verifies that in addition to potassium efflux, calcium influx is also a crucial event for NLRP3 inflammasome activation. Because TRPM2 is a non-selective cation channel that allows the passage of both calcium and potassium (Sumoza-Toledo and Penner, 2011), it is possible that TRPM2 deficiency may also affect potassium efflux. However, as the removal of extracellular calcium did not further decrease the particulate-induced NLRP3 inflammasome activation in *Trpm2*^{-/-} BMDMs (**Figure 4.22**), the defect of NLRP3 inflammasome activation in TRPM2-deficient cells is most likely due to impaired calcium influx.

It should be noted that ATP-induced NLRP3 inflammasome activation was also primarily dependent on calcium influx (**Figure 4.34**). However, in contrast to particulate agonists of the NLRP3 inflammasome, ATP uses P2X₇, but not TRPM2, to induce calcium influx and NLRP3 inflammasome activation (**Figure 4.34**). Consistent with this notion, deficiency in P2X₇ did not affect calcium influx triggered by particulate stimuli (**Figure 4.35**). Taken together, these results suggest that calcium influx is a general proximal step during NLRP3 inflammasome activation, and that different activators of the NLRP3 inflammasome may use distinct plasma membrane ion channels to mediate calcium influx.

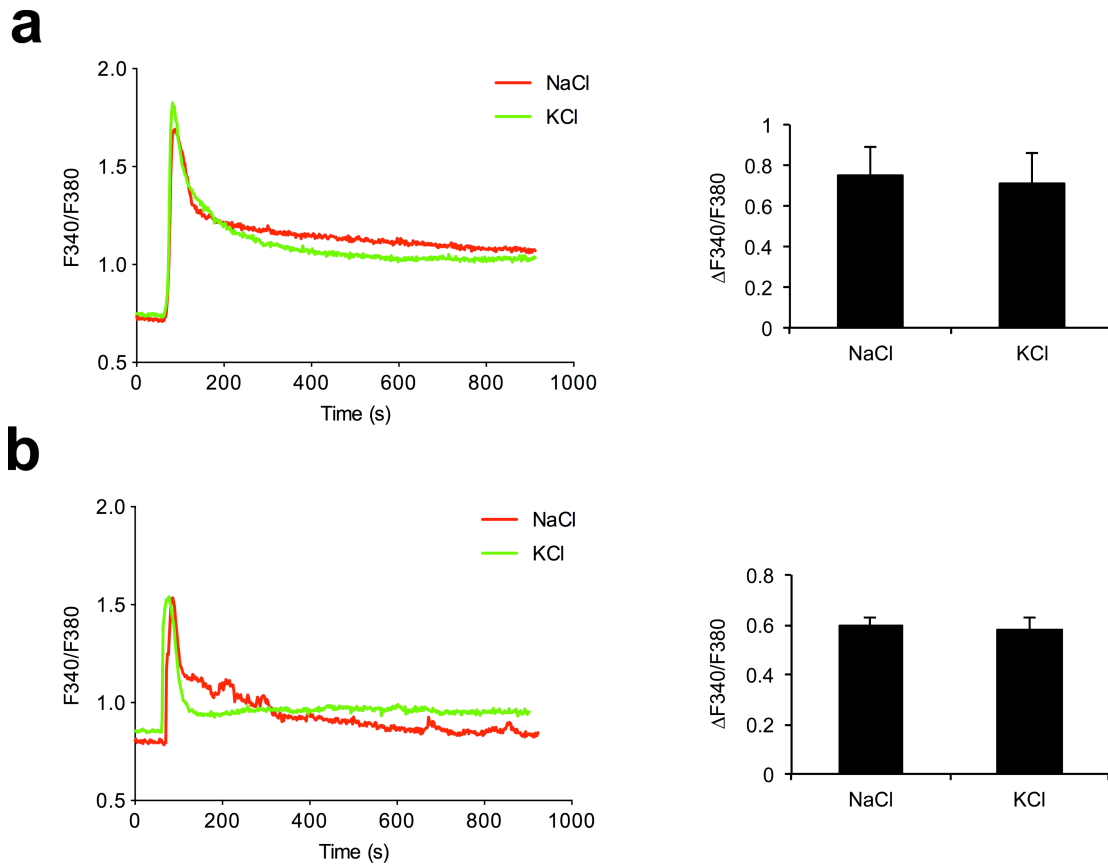


Figure 4.33: Blocking potassium efflux does not affect calcium influx. The maximum $[Ca^{2+}]_i$ elevations, represented by $\Delta F_{340}/F_{380}$, are shown for LPS-primed immortalized murine wild-type or *Nlrp3*^{-/-} macrophages in response to stimulation with liposomes (70 $\mu\text{g/ml}$). The data are shown as mean \pm s.e.m. (n=23-28), and are representative of two independent experiments.

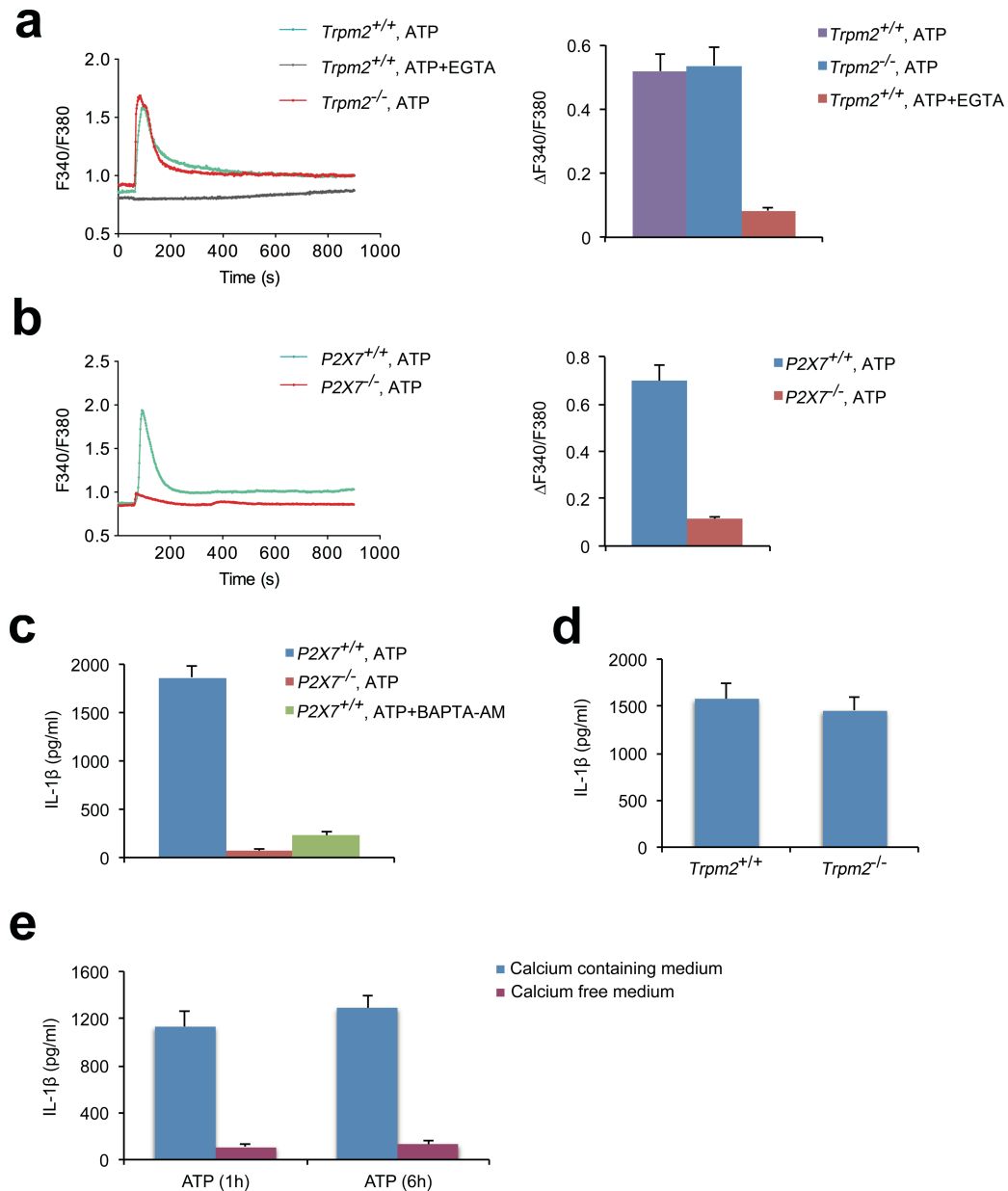


Figure 4.34: ATP activates the NLRP3 inflammasome by inducing Ca^{2+} influx via P2X_7 channel. Time-dependent change of $[\text{Ca}^{2+}]_i$, represented by F340/F380, was shown in LPS-primed *Trpm2*^{+/+} and *Trpm2*^{-/-} BMDMs (**a**, left panel) or *P2X7*^{+/+} and *P2X7*^{-/-} BMDMs (**b**, left panel) cultured in Ca^{2+} -containing solution that were stimulated with ATP (2 mM). The calcium flux over time after ATP stimulation in LPS-primed *Trpm2*^{+/+} BMDMs cultured in calcium-free 0.5 mM EGTA-containing solution was also shown. The maximum $[\text{Ca}^{2+}]_i$ elevations (right panels of **a** and **b**), represented by $\Delta F340/F380$, were shown in LPS-primed BMDMs in response to the ATP stimulation. ATP was

added 1 min after the starting of calcium recording. Data are representative of four independent experiments and are shown as mean \pm s.e.m. in **a** (n=20-36) and **b** (n=22-28). **(c)** ELISA for IL-1 β from supernatants of LPS-primed *P2X₇^{-/-}* or wild-type (*P2X₇^{+/+}*) BMDMs that were pretreated with BAPTA-AM (25 μ M) followed by stimulations with ATP. **(d)** IL-1 β from supernatants of LPS-primed *Trpm2^{+/+}* or *Trpm2^{-/-}* BMDMs that were treated with ATP. **(e)** IL-1 β from supernatants of LPS-primed wild-type BMDMs that were stimulated with ATP in calcium-containing or calcium-free medium. Data are shown as mean \pm s.d. in **c-e**, and are representative of three independent experiments.

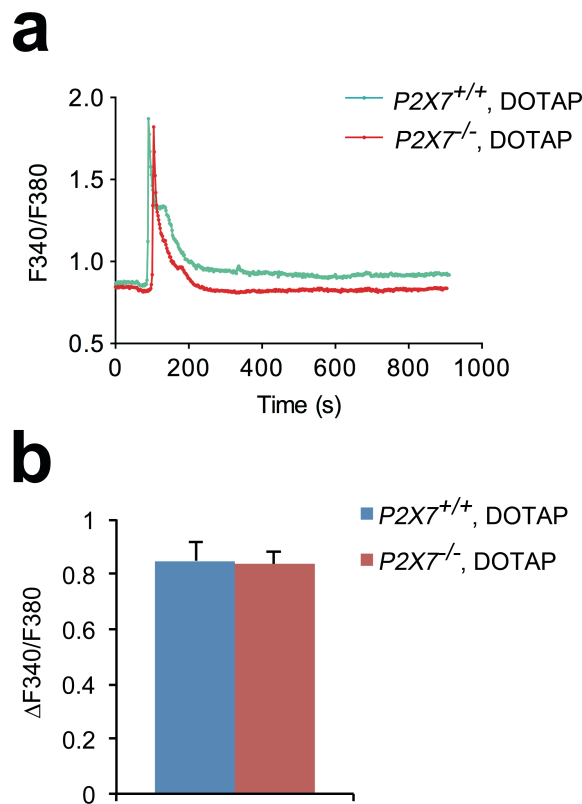


Figure 4.35: Deficiency in P2X₇ does not affect liposome-induced $[Ca^{2+}]_i$ elevation. **(a)** Time-dependent change of $[Ca^{2+}]_i$, represented by F340/F380, was shown in LPS-primed *P2X₇^{+/+}* and *P2X₇^{-/-}* BMDMs cultured in calcium-containing solution that were stimulated with DOTAP liposomes (30 μ g/ml). DOTAP liposomes were added 1 min after the starting of calcium recording. **(b)** The maximum $[Ca^{2+}]_i$ elevations, represented by $\Delta F340/F380$, were shown in LPS-primed BMDMs in response to the stimulation as described in **a**. Data are representative of two independent experiments, and are shown as mean \pm s.e.m. in **b** (n=25-36).

TRPM2 is crucial for NLRP3 inflammasome activation *in vivo*

To further validate the role of TRPM2 in mediating NLRP3 inflammasome activation *in vivo*, we determined whether TRPM2 deficiency affected crystal-/liposome-induced IL-1 β secretion and subsequent neutrophil recruitment after intraperitoneal injections of these particulate stimuli. We first observed that the level of IL-1 β in the peritoneal fluid of the *Trpm2*^{-/-} mice was significantly reduced as compared to that from wild-type mice after injection with either MSU crystals or DOTAP liposomes (**Figure 4.36**). Moreover, particle-induced neutrophil recruitment was also significantly decreased in *Trpm2*^{-/-} mice, which was similar to that observed in *Il1r1*^{-/-} mice (**Figure 4.37**). In contrast, neutrophil recruitment was not affected by either TRPM2 or IL-1R deficiency in response to an intraperitoneal challenge with zymosan, a Toll-like receptor 2 agonist (**Figure 4.38**). These data collectively indicate that TRPM2 is essential for NLRP3 inflammasome activation *in vivo*.

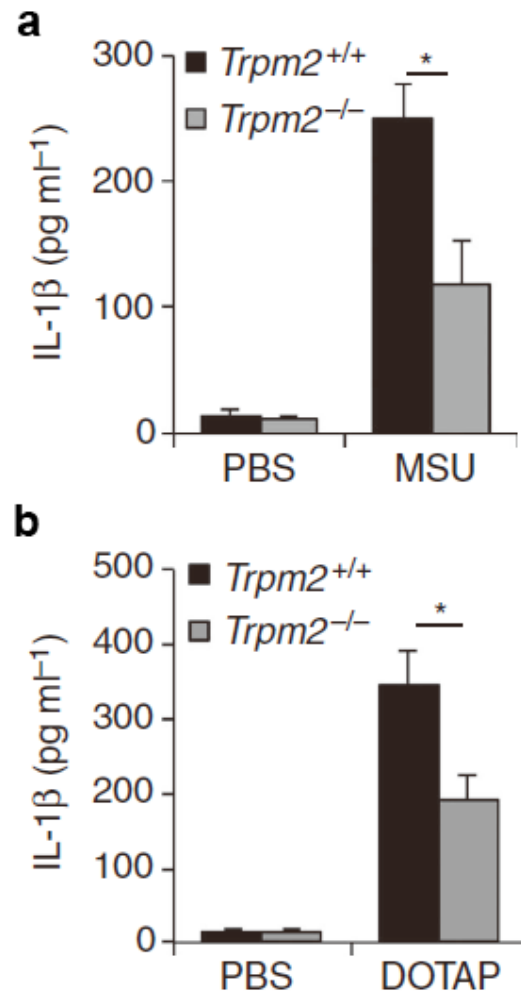


Figure 4.36: TRPM2 is critical for particle-induced IL-1 β release *in vivo*. The IL-1 β concentration (**a**, **b**) were quantified in peritoneal lavage fluid from wild-type (*Trpm2*^{+/+}), or *Trpm2*^{-/-} mice 6 h after intraperitoneal injection of PBS supplemented with either MSU crystals (**a**) or DOTAP liposomes (**b**). The data are representative of two independent experiments (mean and s.e.m. of three to five mice per group). *, $p < 0.05$ versus controls. Statistical significance was determined by the standard two-tailed Student's *t*-test.

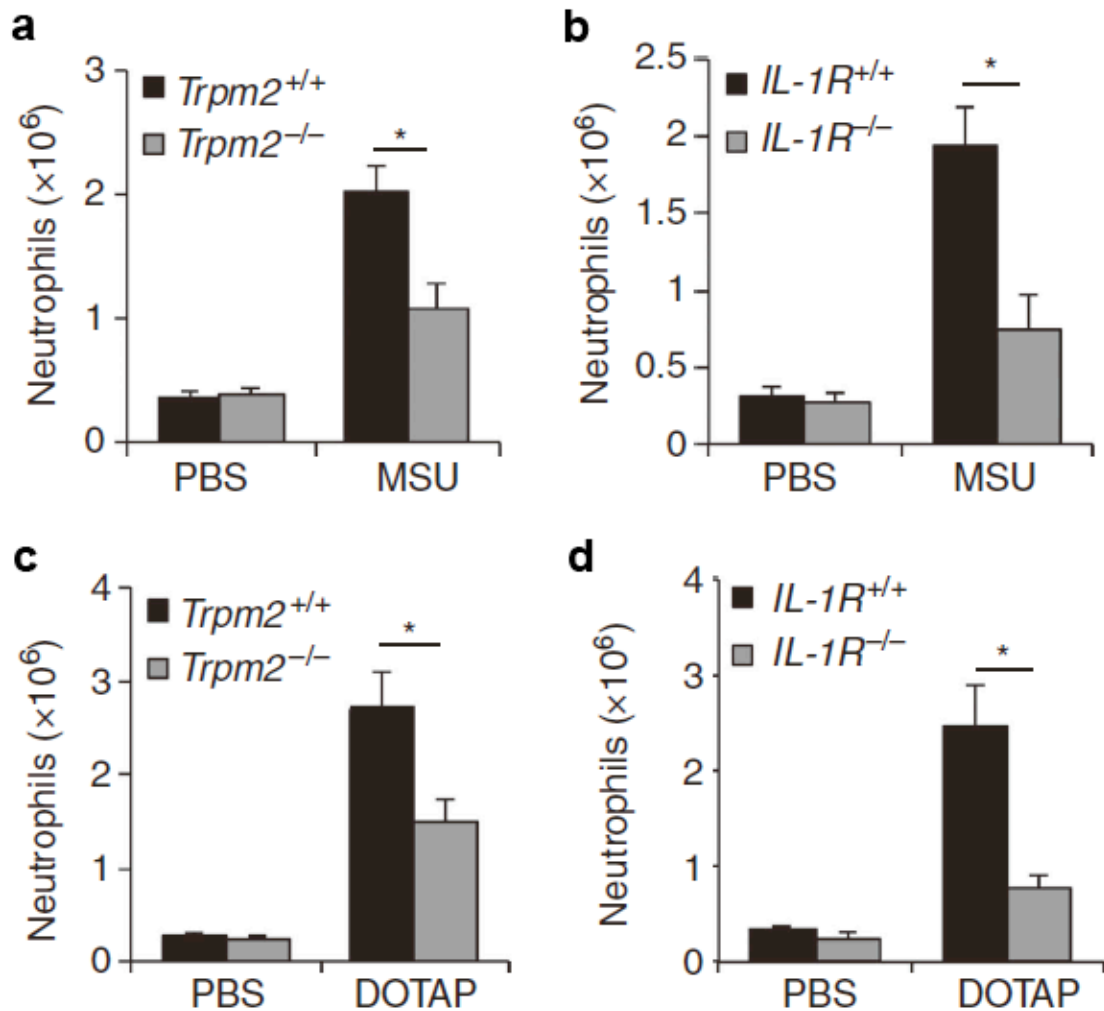


Figure 4.37: TRPM2 and IL-1R mediate particle-induced peritonitis. The neutrophil recruitment (**a-d**) were quantified in peritoneal lavage fluid from wild-type (*Il1r1*^{+/+} and *Trpm2*^{+/+}), *Il1r1*^{-/-} or *Trpm2*^{-/-} mice 6 h after intraperitoneal injection of PBS supplemented with either MSU crystals (**a, c**) or DOTAP liposomes (**c, d**). The data are representative of two independent experiments (mean and s.e.m. of three to five mice per group). *, $p < 0.05$ versus controls. Statistical significance was determined by the standard two-tailed Student's *t*-test.

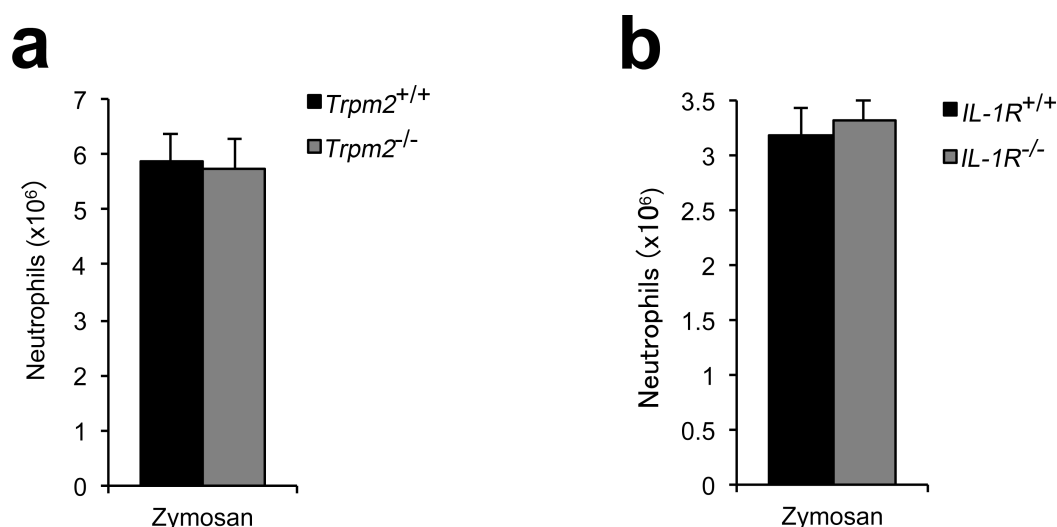


Figure 4.38: Deficiency in TRPM2 or IL-1R does not affect zymosan-induced peritonitis. IL-1 β concentrations (a) and neutrophils influx (b) were quantified in peritoneal lavage fluid from wild-type (*Il1r1*^{+/+} or *Trpm2*^{+/+}), *Il1r1*^{-/-} or *Trpm2*^{-/-} mice 6h after intraperitoneal injection of PBS (0.5 ml) supplemented with zymosan (0.2 mg). Data are representative of two independent experiments (mean and s.e.m. of five mice per group).

TRPM2 is crucial for the adjuvant effect of liposomes *in vivo*

Finally, we tested whether deficiency in TRPM2, which results in decreased IL-1 β secretion, would affect the adjuvanticity of liposomes *in vivo*. As shown in **Figure 4.39**, the level of ovalbumin (OVA)-specific IgG1 was significantly reduced in *Trpm2*^{-/-} mice after immunization with OVA-encapsulated DOTAP liposomes. However, in contrast to the liposomal adjuvant, a deficiency in either TRPM2 did not impair the optimal levels of anti-OVA antibodies when LPS, a Toll-like receptor 4 agonist, was used as the adjuvant (**Figure 4.39**), which suggests that TRPM2- and IL-1R-deficient mice do not have a general defect in mounting an antibody response. Taken together, these data

indicate that TRPM2 and IL-1R are essential for the induction of an optimal antibody response against the antigens that are encapsulated within liposomes.

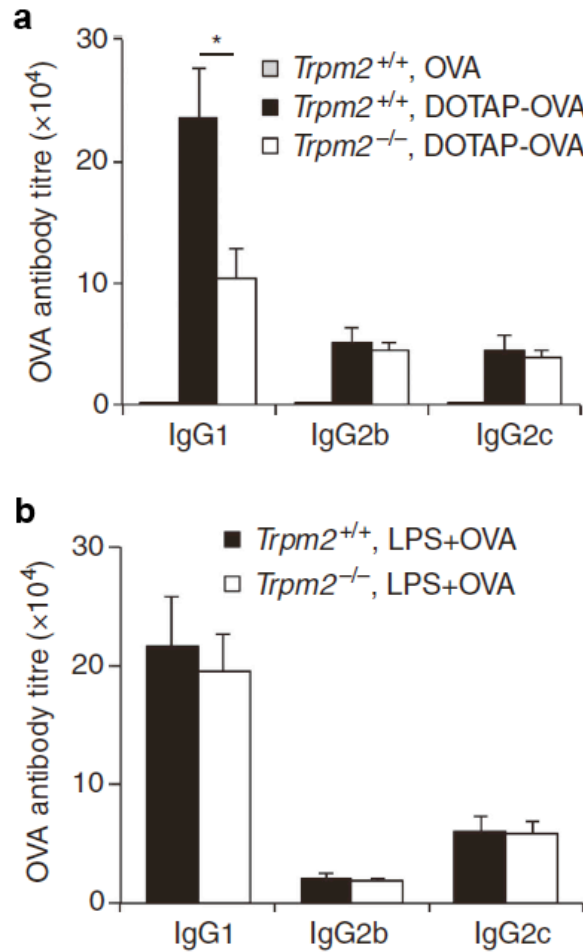


Figure 4.38: TRPM2 is critical for liposome's adjuvanticity. Six- to eight-week old female wild-type (*Trpm2*^{+/+}) or *Trpm2*^{-/-} mice were subcutaneously immunized on day 0 and day 14 with 40 µg/mouse ovalbumin (OVA) alone or the same amount of OVA encapsulated within DOTAP liposomes (**a**) or mixed with LPS (25 µg/mouse, **b**). On day 24, the mice were sacrificed, and sera were collected and analyzed for OVA-specific IgG1, IgG2b, and IgG2c levels by ELISA. The data are shown as geometrical mean ± s.e.m., and are representative of at least two independent experiments (n=4-5 mice per group). *, p<0.05 versus controls. Statistical significance was determined by the standard two-tailed Student's *t*-test.

CHAPTER 5

DISCUSSION

Liposomes are promising immune adjuvants and delivery vectors for the treatment of both infectious and cancerous diseases(Allison and Gregoriadis, 1974; Christensen et al., 2007; Slingerland et al., 2012). Therefore, understanding the innate immune recognition process for liposomes will not only mechanistically define/improve their adjuvant effect but also help to prevent any undesirable inflammatory responses, particularly when liposomes are used as drug-delivery vehicles for anti-cancer therapies. In this thesis study, I identified the NLRP3 inflammasome as a novel innate immune sensor for liposomes. Notably, only the charged, but not neutral, liposomes activated the NLRP3 inflammasome, which is most likely due to the fundamental differences in their induction of mitochondrial ROS. Because the plasma membrane potential is tightly associated with the oxidative state in phagocytes(DeCoursey, 2010; DeCoursey et al., 2003; Murphy and DeCoursey, 2006), it is plausible that charged, but not neutral, liposomes could induce cellular oxidative stress via alteration of the plasma membrane potential, which would eventually result in NLRP3 inflammasome activation. Therefore, charged liposomes may be more suitable as immune adjuvants, whereas neutral liposomes may be more suitable as delivery vectors for anti-cancer drugs.

We also performed additional experiments to test whether the extent of NLRP3 inflammasome activation depends upon the dose and size of the liposomes. As expected,

charged liposomes induce IL-1 β secretion in a dose dependent manner. Interestingly, the size of liposome also determined the amount of IL-1 β secretion. It seems that, within the range of 100nm to 800nm in diameter, the liposome-induced IL-1 β secretion displayed a liner positive correlation with the size of liposomes.

To mechanistically understand how liposomes activate the NLRP3 inflammasome, we first tested if potassium efflux, a seemingly obligatory event for NLRP3 inflammasome activation by essentially all its known activators, was required for liposome-induced inflammasome activation. Indeed, similar to all other known activators, the inhibition of potassium efflux completely abolished NLRP3 inflammasome activation by liposomes. We next tested whether the uptake of liposomes is a necessary step for NLRP3 inflammasome activation. Indeed, we found that the inhibition of phagocytosis in macrophages drastically impaired liposome-induced NLRP3 inflammasome activation. This is likely due to that the uptake of liposomes is required to induce a stress signal that can be sensed by the intracellular innate immune receptor, NLRP3. Interestingly, although the initial phagocytic pathway is required, we found that the lysosome-cathepsin B pathway seems dispensable for liposome-induced NLRP3 inflammasome activation because the inhibition of lysosome acidification or genetic blockade of cathepsin B activity did not significantly affect liposome-induced NLRP3 inflammasome activation. This suggests that the signals necessary for directing NLRP3 inflammasome activation by liposomes appear to be independent of lysosome-derived contents.

ROS has been proposed to be a key player that drives NLRP3 inflammasome activation by most activators. It has been demonstrated that artificially induction of ROS,

in the absence of NLRP3 inflammasome agonists, can trigger spontaneous NLRP3 inflammasome activation and subsequent proinflammatory cytokine secretion. Moreover, the blockade of ROS generation from mitochondria by pharmacological or genetic means can strongly inhibit NLRP3 inflammasome activation by a number of stimuli. It appears that ROS plays a central role in orchestrating the immune responses by activating the NLRP3 inflammasome.

Although an essential role for mitochondrial ROS has recently been implicated in activation of the NLRP3 inflammasome (Kepp et al., 2011; Tschopp, 2011), the precise mechanism underlying ROS-induced inflammasome activation remains elusive. In this thesis study, I identified a novel signaling axis originating from ROS production, followed by TRPM2-mediated calcium influx and ultimately activation of the NLRP3 inflammasome. Therefore, these findings provide at least one mechanistic link that connects oxidative stress with NLRP3 inflammasome activation. Furthermore, the identification of TRPM2 as the responsible channel for calcium influx during NLRP3 inflammasome activation also further advanced our understanding of how calcium mobilization occurs upon stimulation with NLRP3 agonists.

Besides TRPM2, a previous study has also identified TXNIP (thioredoxin-interacting protein) as another link between oxidative stress and NLRP3 inflammasome activation. Upon stimulation with NLRP3 agonists, TXNIP disassociates with its binding protein TRX (thioredoxin) which allows the direct binding of TXNIP to NLRP3. However, TXNIP does not seem to be a universal regulator of NLRP3 inflammasome because several recent studies have shown that TXNIP is dispensable for NLRP3

inflammasome activation by other stimuli. Nonetheless, further studies are indeed necessary to delineate the possible relationship between TNXIP, TRPM2, and NLRP3 inflammasome activation. It would be interesting to see if genetic ablation of TXNIP may affect TRPM2 mediated calcium influx. Alternatively, calcium mobilization might also be a prerequisite for TXNIP to interact and activate NLRP3.

In addition to TRPM2-dependent IL-1 β release, we observed that a portion of particle-induced IL-1 β secretion was independent of TRPM2-mediated calcium influx but instead was dependent on calcium release from intracellular stores. This observation is consistent with a recent report by Murakami *et al.* that demonstrated a role for IP₃R-mediated calcium release from the ER in activation of the NLRP3 inflammasome (Murakami *et al.*, 2012). Additionally, two recent studies have also demonstrated that extracellular calcium elevation, which is a common phenomenon under local inflammation when cell's integrity is compromised, can serve as a danger signal which ultimately induces IP₃R-mediated calcium release from ER to activate the NLRP3 inflammasome.

Although it is clear that both calcium influx and ER calcium release are critical for IL-1 β secretion, how multiple calcium mobilization events cooperatively trigger NLRP3 inflammasome activation remains to be further defined. Recently, a report by Zhou *et al.* demonstrated a close interaction between the ER and mitochondria in response to NLRP3 inflammasome stimuli. Because calcium overload in mitochondria is essential for ROS production, it is plausible that ER-mitochondria contact may be beneficial for rapid calcium flow from the ER into mitochondria, which may facilitate the

generation of sufficient mitochondrial ROS, particularly at later stage during activation of the NLRP3 inflammasome. Moreover, Murakami *et al.* have also demonstrated that in addition to facilitating mitochondrial ROS production, ER-derived calcium is also essential for mitochondria to maintain a damaged state (Murakami *et al.*, 2012). Mitochondrial damage has been shown to induce the release of mitochondrial DNA (mtDNA) into the cytosol, which also activates the inflammasomes (Nakahira *et al.*, 2011; Shimada *et al.*, 2012). In addition to mtDNA, our study demonstrates that ROS released from the damaged mitochondria activate the TRPM2 channel, which results in calcium influx across the plasma membrane. Because blocking TRPM2-mediated calcium mobilization drastically impaired caspase-1 activation and did not affect mitochondrial ROS production, it is possible that, unlike ER-derived calcium, TRPM2-mediated calcium influx may directly signal activation of the NLRP3 inflammasome.

Based on our current study and a previous report by Murakami *et al.* (Murakami *et al.*, 2012), it appears that calcium mobilization is a critical step during NLRP3 inflammasome activation. Calcium is a key signaling mediator for a number of cellular proteases, protein kinases and phospholipases, some of which may be involved in NLRP3 inflammasome activation. Consistent with this notion, ERK1/2 kinase has been implicated in regulating activation of the NLRP3 inflammasome (Cruz *et al.*, 2007). Similarly, we have preliminary data indicating that the inhibition of protein kinase C α also partially impairs NLRP3 inflammasome activation (unpublished results). These calcium-dependent enzymes may function to either proteolytically inactivate a negative

regulator or activate a positive regulator of NLRP3, resulting in the assembly of the inflammasome complex.

Finally, accumulating evidence has suggested that dysregulation of the NLRP3 inflammasome is tightly associated with many inflammatory disorders, such as gout, atherosclerosis, silicosis, asbestosis, and Alzheimer's disease(Davis et al., 2011; Strowig et al., 2012). In this study, we demonstrated that TRPM2 deficiency drastically impairs the inflammatory response induced by MSU crystals, the casual agent of gout. This finding has therefore suggested a therapeutic potential of targeting TRPM2 to treat NLRP3 inflammasome-associated autoinflammatory diseases. Furthermore, other studies have also linked the NLRP3 inflammasome to a number of metabolic diseases, including obesity and type 2 diabetes(Stienstra et al., 2010; Vandanmagsar et al., 2011; Wen et al., 2011). For instance, NLRP3 deficiency protects mice from high-fat diet-induced obesity, and the saturated fatty acid palmitate, which is found in high-fat diets, can induce NLRP3 inflammasome-mediated IL-1 β secretion, which interferes with insulin signaling and eventually results in reduced glucose tolerance and insulin sensitivity. Because our results indicate a role for TRPM2 in NLRP3 inflammasome activation, blocking TRPM2 would be expected to rescue metabolic disorders that are a result of a high-fat diet. In support of this hypothesis, a recent study suggested that TRPM2-deficient mice have attenuated obesity-mediated inflammation and that TRPM2 deletion can protect mice from developing diet-induced obesity and insulin resistance(Zhang et al., 2012). Together, these studies highlight the therapeutic potential of targeting TRPM2 to treat

autoinflammatory and metabolic disorders associated with undesirable activation of the NLRP3 inflammasome.

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VITA

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